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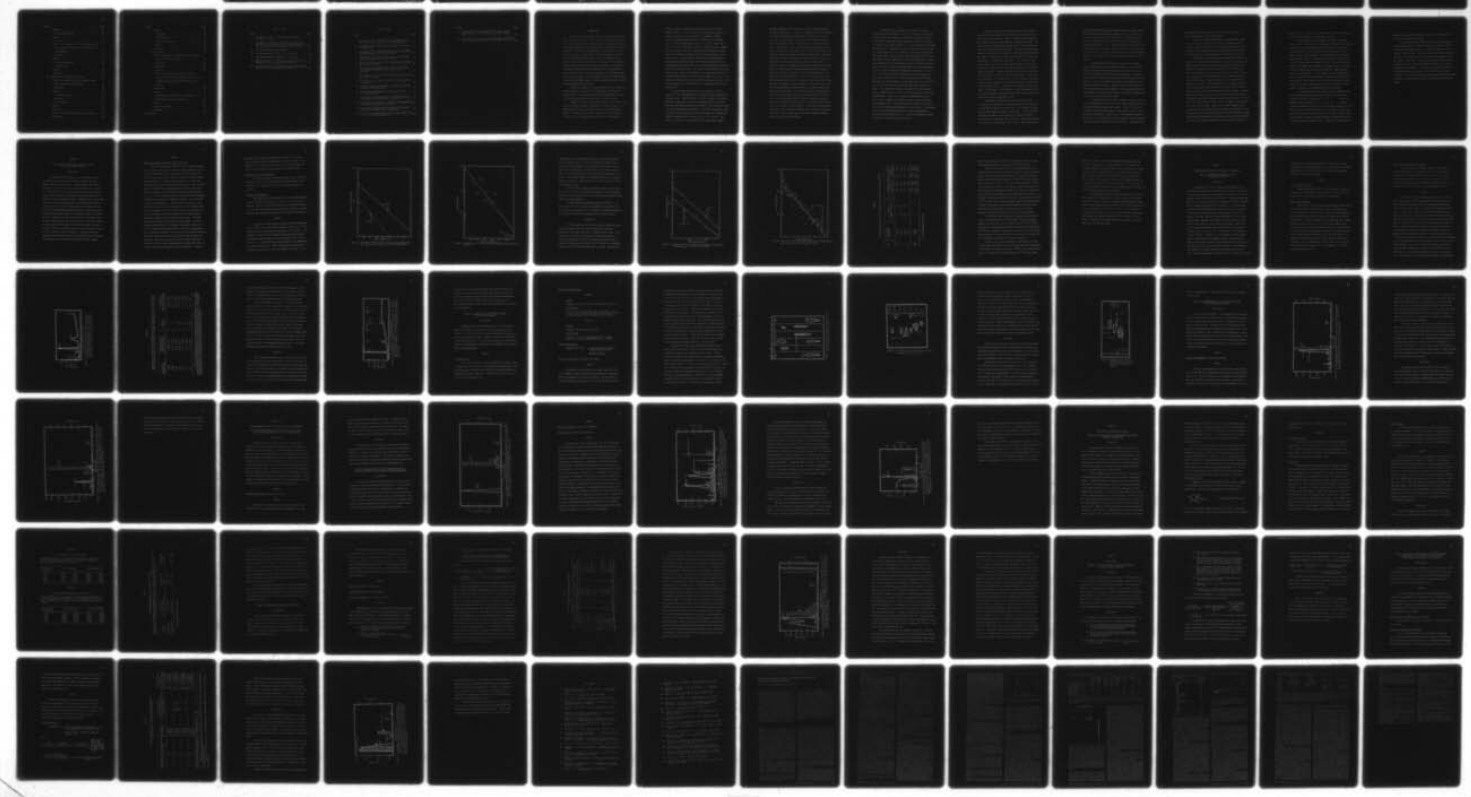

ASCORBIC ACID, BIOLOGICAL FUNCTION AND CHEMISTRY

ANNUAL PROGRESS REPORT

Project Director:
Bert M. Tolbert, Ph.D.

August 1978
(For the Period 1 May 1975 to 3 June 1976)

Supported by

U.S. ARMY MEDICAL RESEARCH & DEVELOPMENT COMMAND
Fort Detrick
Frederick, Maryland 21701

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Department of Chemistry
University of Colorado
Boulder, Colorado 80309

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

INTRODUCTION

This is a report on the concluding year of a study in depth on the chemistry and metabolism of ascorbic acid and isoascorbic acid or erythorbic acid. The broad objectives of the research were to study in detail the biochemistry of these compounds to provide the fundamental background for further studies on the nutritive and stress requirement for the soldier for vitamin C. A further aspect of the study was to determine the metabolic interaction of ascorbic acid and erythorbic acid, since the latter is an optical isomer of vitamin C and also a common food additive, especially in military emergency rations.

During the period of this report, three major projects were studied - the first was the nature and enzymic properties of ascorbate sulfatase; the second was whether C-6 oxidation of ascorbic acid was a significant process in ascorbic acid metabolisms; and the third was an effort to prepare a C-6 oxidized ascorbic acid by synthetic methods, so that its presence or absence in biological tissue could be tested.

REPORT ON ASCORBATE SULFATE SULFOHYDROLASE

Ascorbate is a ubiquitous metabolite of ascorbic acid in higher animals. It is a vitamin in several species of fish. But most important of all, it is stable to air oxidation and does not hydrolyze at neutral pH's. Thus it has special nutritive value for fish. Man surely ingests a considerable amount in his diet.

If ascorbate sulfate is to serve as a source of ascorbic acid there should be an enzyme that can hydrolyze this compound back to ascorbic acid. We have discovered such a compound in a number of animals, and examined in detail the properties of the enzyme from cow liver. This report is attached.

Two interesting questions remain: ascorbate sulfatase is very similar to an important animal enzyme called aryl sulfatase A. The absence of aryl sulfatase A in humans results in the genetic disease metachromatic leukodystrophy. What is the relationship between these enzymes and what is their metabolic role? The other problem is whether ascorbate sulfatase serves to hydrolyze any significant amount of ascorbate sulfate, and whether the ascorbate sulfate has a biological role. At present, it seems ascorbate is both an excretion form of ascorbic acid, and has biological function, perhaps as an hypolipodemic agent.

REPORT ON C-6 OXIDATION OF ASCORBIC ACID

Whether there is significant C-6 oxidation of ascorbic acid in higher animals was tested in two ways. First, a periodate degradation for ascorbic acid was developed, see attached reprint (1976) and this method was applied to urine of monkeys and rats given [6-^{14}C] - ascorbic acid. The experiments, see attached reprint, showed that about 45% of all ascorbic acid metabolites were no longer in the $-CH_2OH$ oxidation state, characteristic of ascorbic acid.

To confirm this result [6-^{3}H] - ascorbic acid was injected into monkeys and their excretion of ^{3}H in urine measured. The excretion was determined for ^{3}H-water in the urine and for organic bound ^{3}H in urine. This study, see attached reprint, showed that again about 45% of the tritum had been released from the ascorbic acid metabolites and appeared in the urine.

The biological significance of this side chain metabolism of ascorbic acid could be related to some special function of ascorbic acid - vitamin C.

PREPARATION OF SACCHAROASCORBIC ACID

If ascorbic acid is subject to C-6 oxidation, a likely product is ascorbic acid with a side chain terminal carbon oxidized to a carboxyl group, or saccharoascorbic acid. Preparation of this compound was attempted in various ways. Success was finally achieved using the method shown in Figure 1. Details of the procedure were published in 1978.

Harkrader, R.J., Plunkett, L.M., and Tolbert, B.M., "Periodate Degradation of Labeled Ascorbic Acid," Analytical Biochemistry 72, 310-314 (1976).

About a month after the new contract year began, October 15, 1975, support for this work was terminated effective January 15th. This date was later extended to June 30th with no additional funds to allow a more orderly termination of the graduate students who were doing the work described in this report.

Their work has been of outstanding quality and their results have been the basis of significant further studies on the metabolism of ascorbic acid. The problem remains to obtain solid scientific results from which optimum intakes of ascorbic acid can be determined for the soldier under various conditions to maximize his ability as a military man.

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This is a final report on the chemistry and metabolism of ascorbic acid. Topics covered in detail include: metabolites in urine and saliva, enzymology of ascorbate sulfatose, synthesis of saccharoascorbic acid, periodate degradation of ascorbic acid and C-6 oxidation of ascorbic acid.

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ASCORBIC ACID, BIOLOGICAL FUNCTION AND CHEMISTRY

FINAL REPORT

Project Director:
Bert M. Tolbert, Ph.D.

August 1978
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Supported by

U. S. ARMY MEDICAL RESEARCH & DEVELOPMENT COMMAND
Fort Detrick
Frederick, Maryland 21701

Contract No. DA-49-193-MD-2611

Department of Chemistry
University of Colorado
Boulder, Colorado 80309

Introduction

This is the final report of a twelve-year study on the chemistry and biochemistry of ascorbic acid Vitamin C. Ascorbic acid is an essential nutrient in the soldier (man) and in addition, recent studies suggest that it has a variety of significant physiological functions that are related to the ability of the soldier to function adequately under a variety of environmental conditions. The nutritional or pharmacological level of ascorbic acid intake for maximum well being in man is a subject of considerable technical controversy and although some of the effects of increased ascorbic acid intake may be small, the possibility that supplemental intake of ascorbic acid can provide improved mental and physiological well being, improved wound healing and recovery from disease, and greater resistance to the debilitating effects of unfavorable environmental conditions, more than justifies military support of research on ascorbic acid.

In addition, scurvy, the overt deficiency disease from inadequate dietary vitamin C, is an important disease of man in war or whenever human populations are displaced from normal food practices. Scurvy is an essential aspect of military nutrition, although it is under excellent control today with synthetic ascorbic acid supplements. That is not to say it will never return, for under the pressure of extended military operations in hostile environments, this nutritional-medical problem can quickly reoccur, even as it has in countless episodes in the history of man.

The discovery and synthesis of ascorbic acid is, comparatively speaking, a relatively modern event achieved in the period 1928 to 1932. The elucidation of the molecular basis of its essential biochemical roles in biological systems has yet to be completed. It is known to be a general oxidizing-reducing compound and to have a critical role in a number of hydroxylation reactions. These functions, or our knowledge of these functions, are not adequate to explain many other facts known about this vitamin. Most important of these facts is the wide distribution, in fact,

ubiquitous presence of ascorbic acid in all multicellular organisms and in unicellular eucaryotes. The role of ascorbic acid in plants, primitive animals, and even algae is unknown. In higher animals, the distribution of ascorbic acid is unique and unexplained. It is present in high levels in such tissue as the brain, eye, gonads and salivary glands, as well as adrenals. The levels of ascorbic acid in higher animals is controlled by several facilitated transport systems and thus rather specifically maintained, suggesting a regulatory role for ascorbic acid. Such a regulatory role for ascorbic acid is also suggested by distribution and levels of this enzyme in plants. One can conservatively state that an adequate understanding of the distribution and function of ascorbic acid in biological systems would provide a scientific basis on which we could develop patterns of use of ascorbic acid for the optimum health and well being of man, including the soldier.

Many scientific approaches to the problem of the essential roles of ascorbic acid in animals have been made. The most common is to carry out a physiological, nutritional, or medical problem *with or without added* ascorbic acid and to measure the magnitude of the effect. In reality, these are serendipitous experiments, but in total, a great number of interesting effects have been reported, and are still being discovered. However, no unifying biochemical theory for their molecular basis has been discovered.

The primary objective of the research supported by this contract has been to workout the biochemistry and metabolism of ascorbic acid and to use this information as a basis for physiological and nutritional studies concerning Vit C in man.

The evolution of the chemical and biochemical problems studied in this research is interesting and informative. They indicate a continuing increase in our knowledge of this vitamin. A summary of the scientific aims for this study for the years 1965 through 1975 is given below, together with current comments.

16 April 1965. Aims. To study oxidation of ascorbic acid (As) to dehydroascorbic acid (dAs) to oxalate on a chemical and enzymic level. Comments. The objective is sound, but oxalate will prove to be a minor metabolite, and tissue levels of dAs very difficult or impossible to measure.

1 June 1966. Aims. Same as above, but broadened to cover sequence of events involved in the in vivo catabolism of ascorbate.

1 May 1967. Aims. Broad aims to cover the biochemistry and enzymology of As metabolism in the guinea pig, rat and human.

10 April 1968. Aims. Same as in 1967, but with the introduction of the concept that biochemical information on ascorbic acid could be used to explore the role of As in the physiology of stress. Comments. Rather complex carbon-14 labelling experiments are proposed.

1 March 1969. Aims. Objective same as in 1967-1968. Comments. The concept is introduced that specific biochemical roles of ascorbic acid are involved in the optimum use of As by animal systems.

28 May 1970. Aims. Same as in 1967-1969. Comments. The concept is introduced that the food additive, D-isoascorbic acid, could have bad effects in human nutrition.

1 April 1971. Aims. Same as in 1967-19[illegible] Comments. The role of ascorbate sulfate, a new metabolite of As in ma[illegible]ed.

1 June 1972. Aims. Same as in 1967-1970 plus [illegible]preparation of ascorbate derivatives for use in metabolic studies.

23 May 1973. Aims. Metabolic studies as before using ^{14}C, ^{3}H, and ^{35}S labeling techniques plus development of analytical methods for As metabolites.

23 May 1974. Aims. As above plus a renewed emphasis on effect of stress on ascorbate metabolism, and the possible effect of isoascorbic acid on As metabolism and utilization. Comments. By now it was recognized that the best animal-experimental results could be obtained by studying these factors in monkeys. The metabolism of As is quite different in primates as compared to rats or guinea pigs.

May 1975. Aims. Same as in 1974. Comments. The early work proposed under this contract was a chemical and enzymic study of the only known metabolites of ascorbic acid in the usual animal used for such studies, the guinea pig. By the termination of this study, it was determined that the guinea pig was not a good animal for studies--they should be done in primates--and that the early metabolites of ascorbic acid proposed for study were minor products, probably of secondary interest, and that the metabolism of ascorbic acid was far more complex than suggested by the literature on

this subject. During this period, Professor Linus Pauling gave dramatic publicity to the concept that a high intake of ascorbic acid had value in the prevention and cure of colds. The publicity on the problem was surely good, and the number of scientific publications on this subject increased several fold. In ways the publicity obscured Pauling's more fundamental point, namely that ascorbic acid had a role in generalized treatment of a stress, a concept that antedated Pauling's public and scientific statements by several decades. Unfavorable publicity concerning Pauling's promotional activities also tended to reflect poorly on a growing body of knowledge that clearly demonstrates a role for ascorbic acid in stress reactions. This aspect of the problem probably remains the one of greatest potential interest to the military, and one in which fundamental research and applied research are both quite appropriate. Afterall, stress is a major factor in the well being of the soldier, both in war and peace time.

During the course of this study, isoascorbic acid has been used many times. It has the same structural formula as ascorbic acid, but differs in being a "diasterioisomer" or an "optical isomer." It is commonly used as a food additive because of its antioxidant properties. While not toxic as a food additive, its use in foods contradicts good biochemical judgment, insofar as many diasterioisomers of essential nutrients are antagonists of the nutrient itself. Work on the metabolic aspects of this problem are important since isoascorbic acid (commercial name: erythorbic acid) is a very common additive in the military ration. This work did not progress very far, but it remains an important problem. More recent studies, some of them currently in progress at the laboratory of the principal investigator, show that major metabolites of ascorbic acid have a chemically modified side chain. This in turn indicates that isoascorbic acid with a D-configuration on the side chain should not be metabolized in a similar way to ascorbic acid, where the configuration is L.

The following sections cover most of the important details of the research that have been completed at this time and not reported in previous annual reports. In addition, several papers have yet to be written. Sections are as follows:

Part I

ASCORBIC ACID METABOLITES IN SALIVA
by Sheryl Joyce Loux
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*As is ascorbic acid; SAs is saccharoascorbic acid.

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INTRODUCTION

Man has a total of about two grams of ascorbic acid (As) and its metabolites in his body. Very little of this amount of ascorbic acid seems to be needed in known and assigned biochemical roles, such as an enzyme cofactor in the hydroxylation of proline and amino acid metabolism. The highest concentrations of As are found in tissues such as the brain, salivary glands, and gonads, where no significant amounts of hydroxyproline synthesis occur. The disturbing fact remains that for most of the As found in the body, the biochemically active form(s) and biochemical role(s) are unknown. The discovery of catabolic products may lead to discovery of a new biologically active form of ascorbic acid. Such an active form of ascorbic acid could, in turn, suggest a possible chemical role for As, thus helping to explain the unknown function of the overwhelmingly greater portion of this essential bodily substance.

Common assay methods for determining As levels in biological tissues are based on one of the following chemical properties of ascorbic acid: 1) the reduction capacity of the enediol group, and 2) the ability of oxidized ascorbic acid to form an osazone.

Methods of employing the reduction capacity of As rely on the detection of those substances reduced by As, such as iodine, phosphomolydate, ferric ions, methylene blue, and dichloroindophenol (DCIP). Of these oxidizing agents, the DCIP test, introduced by Tillmans (1), has the greatest specificity for As in biological

tissues. Bessey (2) showed the highest specificity of the DCIP method for As at pH 3.5. At pH 4.0 naturally occurring phenolic or sulfhydryl groups may react with DCIP. Thiosulfate, sulfite, ferrous, cuprous and stannous salts interfere with the DCIP test (3). At a pH of greater than or equal to 7, DCIP is a dark blue color. At a pH of less than 7, DCIP is a light pink color. Reduced DCIP is colorless at any pH. Visualization of the light pink end-point of a dilute DCIP solution in a DCIP titration assay is uncertain at best. In preference to visualization, spectrophotometers have been used even though many corrections are necessary for the large background absorption and slowly reacting substances associated with most biological tissues. DCIP reacts on an equimolar basis with ascorbic acid. Zannoni (4) developed a more sensitive colorimetric procedure where the ferric ion was reduced to the ferrous state. The reduced iron couples with 2,2'-dipyridyl to produce a red color. The color development is read at 525 nm and is linear with the amount of ascorbic acid.

An assay based on the ability of oxidized As to form an osazone with 2,4-dinitrophenylhydrazine (DNPH) was developed by Roe and Kuether (5). Trichloroacetic acid (TCA) is added to the sample, then the ascorbic acid is oxidized to dehydroascorbic acid usually with Norit and either filtered or centrifuged. The sample may or may not be centrifuged prior to oxidation. The DNPH in 9N H_2SO_4 is added to react with the oxidized As to form an osazone while incubating at elevated temperatures (eg. 3 hours at 37°C, 1 hour at 56°). The samples are cooled in an ice water bath, and the osazone crystals

are slowly dissolved in 80% w/w H_2SO_4. The red color developed is recorded at about 515 nm. The 515 nm absorbance for the As standards is directly proportional to the amount of As present up to about 50 µg (5). The sensitivity of this colorimetric method is second only to the Zannoni method. The specificity of the DNPH method is poor. All five and six carbon sugar-like compounds produce the red color, but this color fades more quickly than with As and the method allows 30 minutes prior to color absorption measurement. Additional interference occurs from the known metabolites--dehydroascorbic acid, As-2-SO_4 (6), and could manifest from other As derivatives. C-6 ascorbate oxidation is a known major metabolic process (7) and could result in such derivatives as saccharoascorbic acid (8), which is shown in this thesis to assay on an equimolar basis with As in the DNPH, DCIP and Zannoni methods. Also, derivatives of As with acid labile groups on the C-2 and C-3 positions could significantly interfere with the DNPH assay after hydrolysis at those positions, since the acid concentration is greater than 3N at elevated temperatures during color development. This interference has been demonstrated with ascorbate-2-sulfate (As-2-SO_4), a known metabolite, by Baker, et al. (6) using the DNPH method. Another problem with the DNPH method is that much biological material is precipitated and removed by either filtration or centrifugation. The extent of precipitation and removal of DNPH material fluctuates due to different analytical procedures. Thus, caution must be used when interpreting "whole" sample analysis by the DNPH method.

In the search for ascorbic acid in biological tissues, saliva has been investigated. The first investigator of ascorbate in human saliva was Stuteville in 1935 (9). Stuteville used the DCIP titration method on paraffin-stimulated and acidified saliva. He reported a measurable level of 2.5 μg ascorbic acid per milliliter of whole saliva. Stuteville also did a biological test with saliva on a single guinea pig. The guinea pig was scorbutic with rapidly declining weight. The animal was given a daily dose of saliva, and the weight loss was stopped with a slight net weight gain. The bioassay suggested that an antiscorbutic factor was present in saliva, but the value of a single animal experiment is highly questionable. Zimmet and Dubois-Ferrière (10) reported an average of 1.42 μg As per ml of saliva in ten human subjects of good health using the DCIP assay. Glavind et al. (11) determined the ascorbic acid concentration by the DNPH method of a stimulated human saliva sample to be 1.7 μg As per ml and of a non-stimulated sample to be 2.4 μg As/ml. In a study of 85 subjects, Hess and Smith (12) reported a mean As concentration of 1.9 μg As/ml for unstimulated saliva using the DNPH test. Also, a mean salivary As concentration of 0.7 μg As/ml for 110 subjects by the DNPH assay was reported by Freeman and Hafkesbring (13). The measurements of ascorbic acid levels in saliva listed above were determined on "whole" saliva. As mentioned previously, the DNPH method necessarily removes precipitated materials to some extent due to the analytical procedure of certrifugation or filtration to remove the Norit.

Whole saliva consists of secretions from two cell types: serous cells which excrete a clear watery fluid, and mucous cells which excrete a thick mucous. There are three salivary glands in man: the parotid, the submaxillary and the sublingual glands. The parotid ducts are located on the cheeks across from the upper back molars and lead from serous cells only. The submaxillary ducts are located at the most forward junction of the tongue with the floor of the mouth. The submaxillary ducts lead from serous cells and some mucous cells. The sublingual ducts open at the junction of the tongue with the floor of the mouth, but are lateral to the tongue on each side. There are also sublingual ducts which exit with the submaxillary ducts. Sublingual glands consist of mucous cells and some serous cells. Saliva is greater than 99% water. The solids of saliva consist of inorganic salts, a small amount of urea, mucin (a glycoprotein), enzymes for polysaccharide hydrolysis and traces of some vitamins (14, 15). Whole saliva samples may contain food debris, oral epithelial cells, bacteria, fluid from pockets of the gums, and blood cells -- any of which might effect the results of an ascorbic acid assay of the whole saliva.

Pretreatment of whole saliva by centrifugation on 25 salivary samples resulted in an average value of 0.93 µg As/ml by the DNPH test as reported by Dreizen (14). The sediment fractions from the above samples averaged 2.16 µg As/ml. Dreizen (14) also found the sediment of centrifuged saliva higher in Vitamin A, pantothenic acid, nicotinic acid, and biotin. Mäkilä and Kirveskari (15) found the sediment higher in folic acid. Odumosu and Wilson

(16), using the DNPH assay, reported that whole saliva after treatment with TCA and centrifugation contains one sixth as much As as found in whole saliva with no pretreatment. Examination of the sediment showed epithelial and mucosal cells, food particles and some polymorphonuclear cells. Selective staining of the cells with silver nitrate was reported to show granules of As in the cytoplasm and cell nuclei (16); however, the specificity of the silver nitrate method for As in biological tissue has not been extensively investigated.

Stimulated parotid saliva was assayed for As using the DNPH test by Bates (17), who reported a value of 1.9 μg As/ml from averaging the results from 66 human males, and a value of 2.3 μg As/ml from averaging the results of 40 human females. Mäkilä and Kirveskari (18) collected parotid saliva and mixed submaxillary and sublingual saliva by olafactory stimulation from subjects who had fasted a minimum of 12 hours. The mean parotid value was 1.26 μg As/ml by the DNPH assay on 13 subjects. The reported value for the mixed submaxillary and sublingual saliva on the same 13 subjects was 0.60 μg As/ml, also by the DNPH method.

In addition to the DCIP, DNPH and Zannoni methods, confirmation of the presence of As in biological tissues by means of chromatography has been attempted more recently. Bates (17) reported he was unable to confirm the presence of free ascorbic acid in saliva by using chromatography. Feller et al. (19) attempted to detect As by paper and gas chromatography using pilocarpine stimulated parotid and whole saliva from dog and saliva from humans. They reported no

detectable free ascorbic acid in saliva, and their recoveries of As when mixed with saliva were excellent.

In contrast, Smith (20) reported in an abstract that human saliva contains 50% ascorbic acid and 50% dehydroascorbic acid, observed by liquid chromatography. Using the Zannoni method, Smith reported a total concentration of the reduced and oxidized forms of As in saliva as being approximately the blood level (10 μg As/ml).

Many investigations have been undertaken to determine whether there is an ascorbic acid dose response in salivary As levels in man. Zimmet and Dubois-Ferrière (10) administered orally 500 mg As and observed no increase in salivary ascorbic acid by the DNPH test. Hess and Smith (12) orally administered 500 mg As dissolved in water and observed that for 3 hours there was no increase in salivary As using the DNPH assay. Dreizen (14) gave 500 mg of ascorbic acid daily for seven days. Salivary samples on the eighth day failed to show a statistically significant rise in ascorbic acid by the DNPH test as compared with the preadministration saliva samples. However, Mäkilä and Kirveskari (18), using the DNPH method, found a parotid As concentration rising trend maximizing in four to eight (4-8) hours after oral administration of 580 mg of As to subjects who had fasted for a minimum of 12 hours. Mäkilä and Kirveskari suggest that a negative result could be expected using whole saliva because of large background levels and possible associated errors mentioned previously, and because the three hour investigation period used by investigators was too short. Dessy and Doneddu (21) noted a slight increase in

saliva As levels 2-3 hours after administration of 1 gram of ascorbic acid given intravenously, but they quoted no figures.

The correlation of saliva ascorbic acid levels with other tissues in man is non-existent. Freeman and Hafkesbring (13) reported that blood, urine and saliva DNPH reactive material concentrations did not correlate. Bicknell and Prescott (22) concluded that the salivary glands secrete ascorbic acid independently of the levels in other tissues. Bates (17) investigated the levels of ascorbic acid in saliva, plasma and white blood cells (WBC). The saliva and WBC ascorbic acid levels were estimated using the DNPH assay. The plasma levels of As were determined by the DCIP method. Bates reported a significant correlation ($P < 0.001$) between the As concentration of saliva and that of WBC in young (dental students) and old (over 65) male subjects. Bates did not find this same correlation for female subjects. No other statistically significant correlations of the levels of ascorbic acid in the three fluids was observed.

Low salivary levels of As as measured by the DNPH test have been reported for subjects with pathological conditions in which vitamin C nutrition is considered to be disturbed. Bijos (23) reported low levels of ascorbic acid in the saliva of TB subjects. Zimmet and Dubois-Ferrière (24) observed that some infectious diseases result in low As concentrations in saliva. Post-tonsillectomy subjects were reported to have low ascorbic acid levels in saliva (25). Lower salivary As values have been reported in patients with caries as compared to healthy subjects (10). However, Hess and Smith (12)

observed no significant difference in salivary As concentrations in carious and noncarious patients.

Lars Hammarström (26) reported on the salivary gland (sublingual and submaxillary glands) distribution patterns of 1-^{14}C-As as determined by impulse counting. Hammarström injected 1-^{14}C-As in mice by the tail vein. Data was obtained by organ extraction and counting. At 5 minutes, the time of the first measurement, there was already a high level of ^{14}C in the submaxillary gland, and the radioactivity in this gland considerably decreased by the second measurement at 30 minutes. In contrast, the sublingual gland had a slower uptake, which maximized at 4 hours after the radioactive injection. A 5-minute autoradiogram clearly showed the faster uptake of label by the submaxillary gland as compared with the sublingual gland.

Chapter I

^{14}C-As LABELING OF THE RATS: SPECIFIC ACTIVITY OF ^{14}C IN SALIVA AND URINE

Introduction

Literature reports have given some indication that the frequently used wet chemical As assays are reacting with some material other than As in saliva. Numerous papers report levels of ascorbic acid in human saliva of about 0.6-2.5 μg/ml (9, 10, 11, 12). These levels of As in saliva are obtained using wet chemical assays, namely the DNPH, Zannoni and DCIP methods, thought to be specific for As in biological tissues. However, Feller, et al. (19), using paper and gas chromatography, found no detectable levels of ascorbic acid in pilocarpine stimulated saliva in dog, but did report using the dinitrophenylhydrazine (DNPH) wet chemical As assay, a DNPH reactive material equal to a standard As value of 0.9 μg/ml. In light of Feller's work and the interference in the DNPH assay of the known metabolite As-2-SO_4, the specificity of the DNPH, Zannoni, and DCIP assays for detecting As in biological tissues is suspect. Therefore, ^{14}C-As was chosen to be used as a highly specific analytical tool to detect As and As metabolites. Rats were chosen for radioactive labeling because they were available and easily handled. The radioactive labeling of rats is described in this section.

Methods

Radioactive Labeling of Rat and Saliva Collection

A glass vial containing solid ^{14}C-As in a vacuum was opened. Enough double-distilled sterile water was added to make an isotonic solution and dissolve the As. Immediately, the dissolved 1-^{14}C-ascorbic acid with a specific activity of 50 μCi/10 mg was injected subcutaneously (s.c.) into an albino female rat. In some cases, the radioactive vial was rinsed with sterile saline. The vial rinse was withdrawn with the same syringe containing the original radioactive As solution. This radioactive solution was then injected s.c. The vial was rinsed with saline a second time and the contents withdrawn into the original radioactive syringe. This second vial rinse was also injected s.c. The volume of the second vial rinse was at least equal to the sum of the volumes of the original radioactive As solution and first vial rinse solution. Just prior to saliva collection, the rat was inserted into a slightly elastic cloth tube equipped with neck and tail draw strings. This tube allowed easy breathing but virtually no leg movement. A sterile isotonic water solution of pilocarpine, equivalent to about 6.7 x 10^{-3} g pilocarpine HCl/kg body weight, was injected interperitoneally through the cloth tube. The rat was then laid ventral side down upon a rack, which was tilted at about 30° so that the rat's head was down. Whole saliva was aspirated into a small filter flask. Collection of saliva began approximately two minutes after the pilocarpine injection and continued for 20 to 30 minutes. A typical

20-30 minute collection period resulted in about 1 ml of saliva being collected, although sometimes much less was collected. After withdrawal of an aliquot of saliva for scintillation counting, the filter flask was sealed with paraffin and refrigerated at 0°C.

Radioactive Rat Urine Collection

Rats were contained in a glass metabolism cage. Urine was collected in 10 ml of 1.5M ammonium formate adjusted to pH 2 with 88% formic acid. Generally, urine was collected at twelve-hour intervals.

Counting of Radioactivity

The solubilizer for the Beckman LS-250 scintillation counter was 15 ml of [200 g naphthalene; 10 g PPO; 2 liters scintillation dioxane]. 100 µl of freshly collected saliva or urine were counted. 0.5 ml of the liquid chromatograph fractions was counted. Counting efficiency was determined by spiking a sample with a dpm standard.

Results

In the first of two separate long-term labeling experiments using 1-^{14}C-As, rat A (313 g body weight) was given 50 µCi in 10 mg As and rat B (276 g body weight) was given 15 µCi in 3 mg As. The dpm/100 µl of whole saliva collected and the average dpm/hour of urine excreted are shown for both rats on Figure 1 and for Rat A on Figure 2. Rat A received all of the contents of a radioactive vial plus multiple rinses. Rat B received the last portion of a ratioactive solution in a vial plus multiple vial rinses. For the

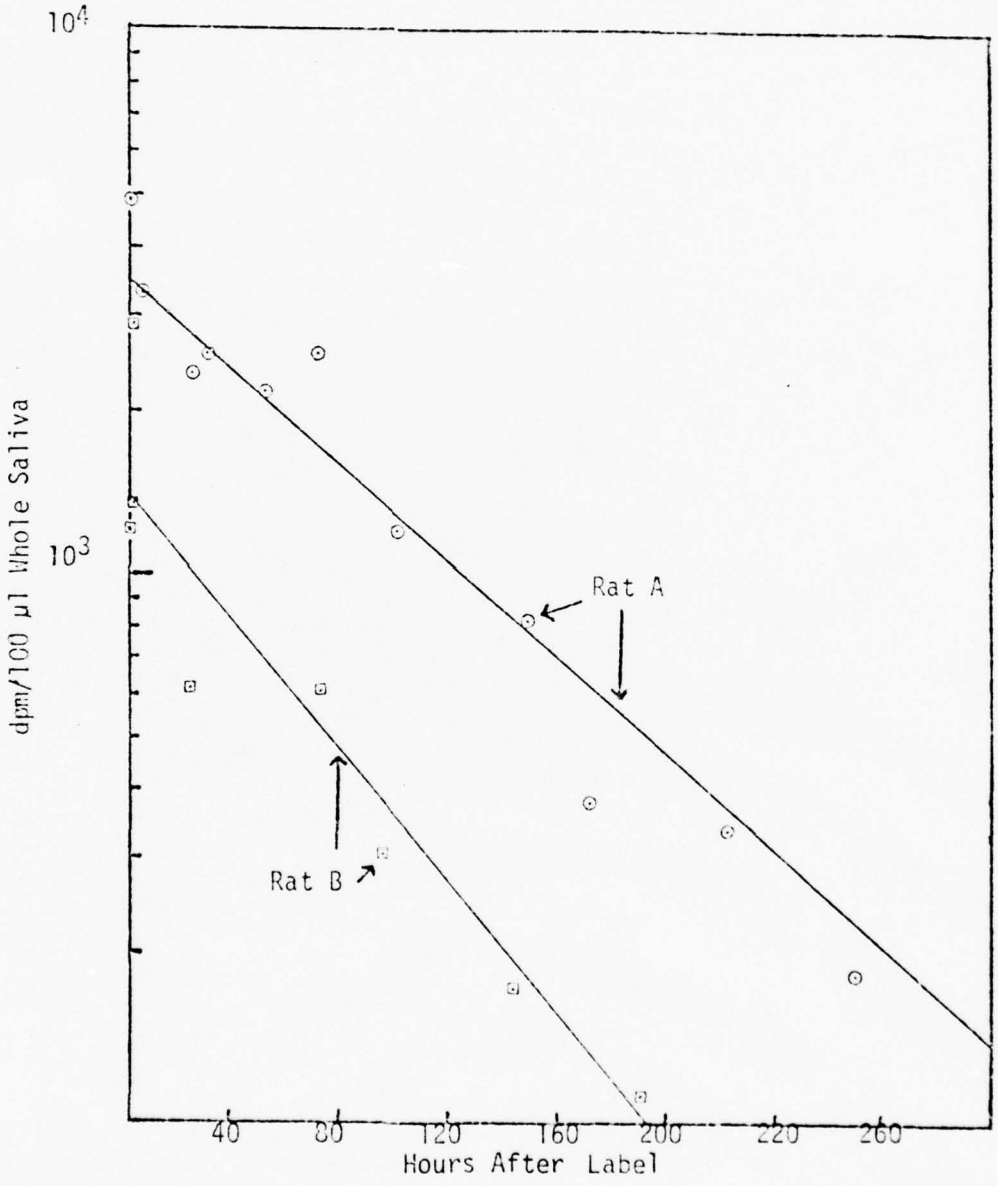


Figure 1 Excretion of ^{14}C in Saliva of Rats A and B Labeled with 50 µCi/10 mg and 15 µCi/3 mg 1-^{14}C-As, Respectively.

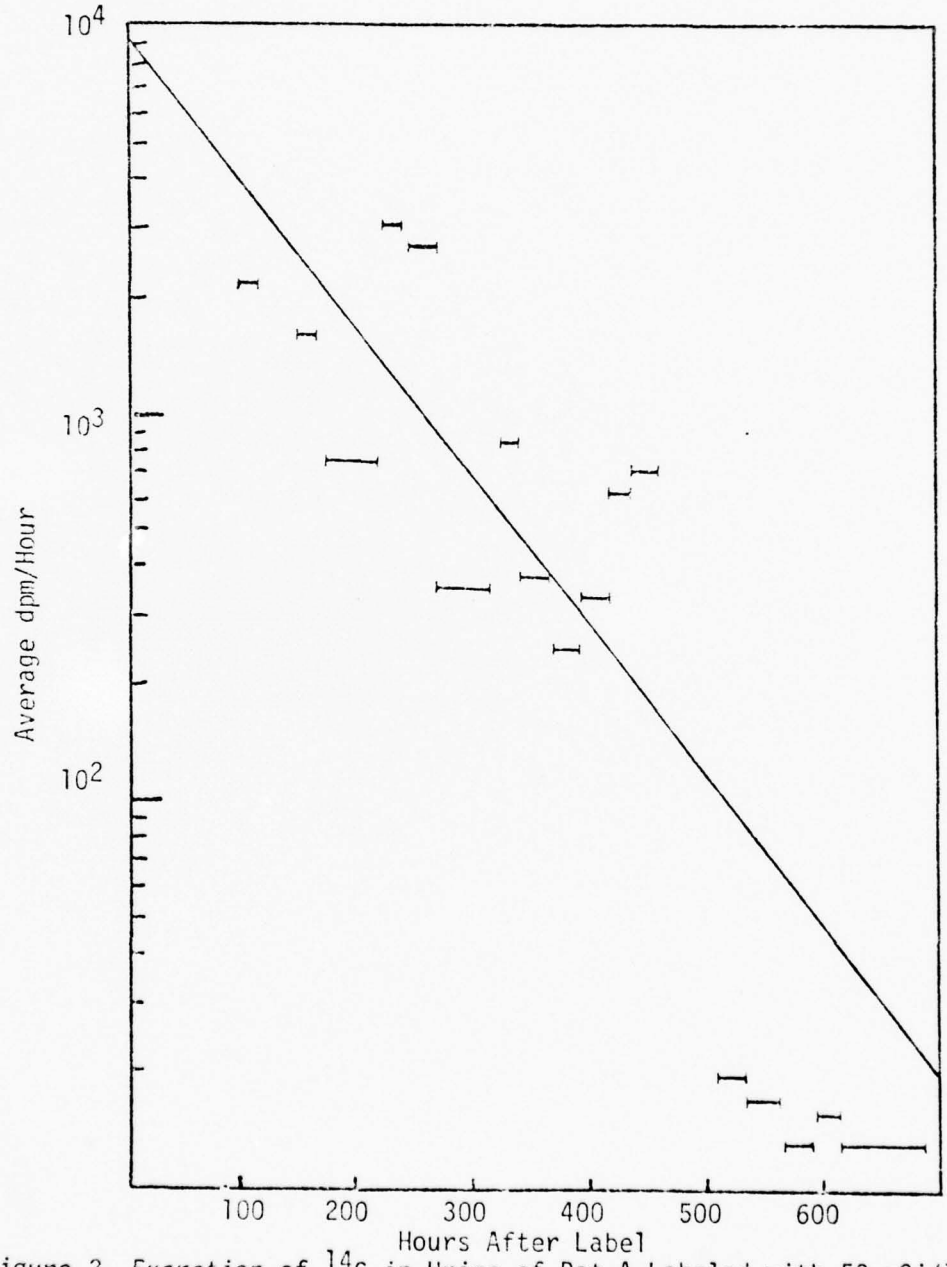


Figure 2 Excretion of ^{14}C in Urine of Rat A Labeled with 50 μCi/10 mg 1-^{14}C-As.

second long-term labeling experiment, the saliva and urine counts for rat A (430 g) and rat C (324 g) are shown in Figures 3 and 4. In this second experiment, rat A received the final portion of the radioactive solution plus multiple rinses of the radioactive vial. Rat C received a measured portion of the radioactive solution and no rinses. The lines of Figures 1-4 are obtained from a least squares analysis of the data. The biological half-life of each set of data is listed in Table I.

A third 1-^{14}C-As labeling experiment, where rat C was given 5 μCi in 1 mg 1-^{14}C-As, resulted in 175 dpm/100 μl of whole saliva. The saliva was collected 35 minutes to one hour after the final radioactive vial rinse injection.

A fourth labeling experiment was done with 6-^{14}C-As. Rat D was given 16 μCi in 4.9 mg As, and the specific activity of the whole saliva was 884 dpm/100 μl. The saliva was collected 0.83 hour to 1.33 hour after the final radioactive vial rinse injection.

Discussion

1-^{14}C-As labeled rats were found to give a radioactive peak in saliva at about one hour after the radioactive injection. Hammarström has studied the levels of labeled ascorbic acid in the salivary glands of mice as a function of time after labeling. A very fast accumulation of ^{14}C in the submaxillary gland (within 5 minutes of labeling) was observed in mice, followed by a subsequent rapid decline of ^{14}C in this gland after 5 minutes. The sublingual

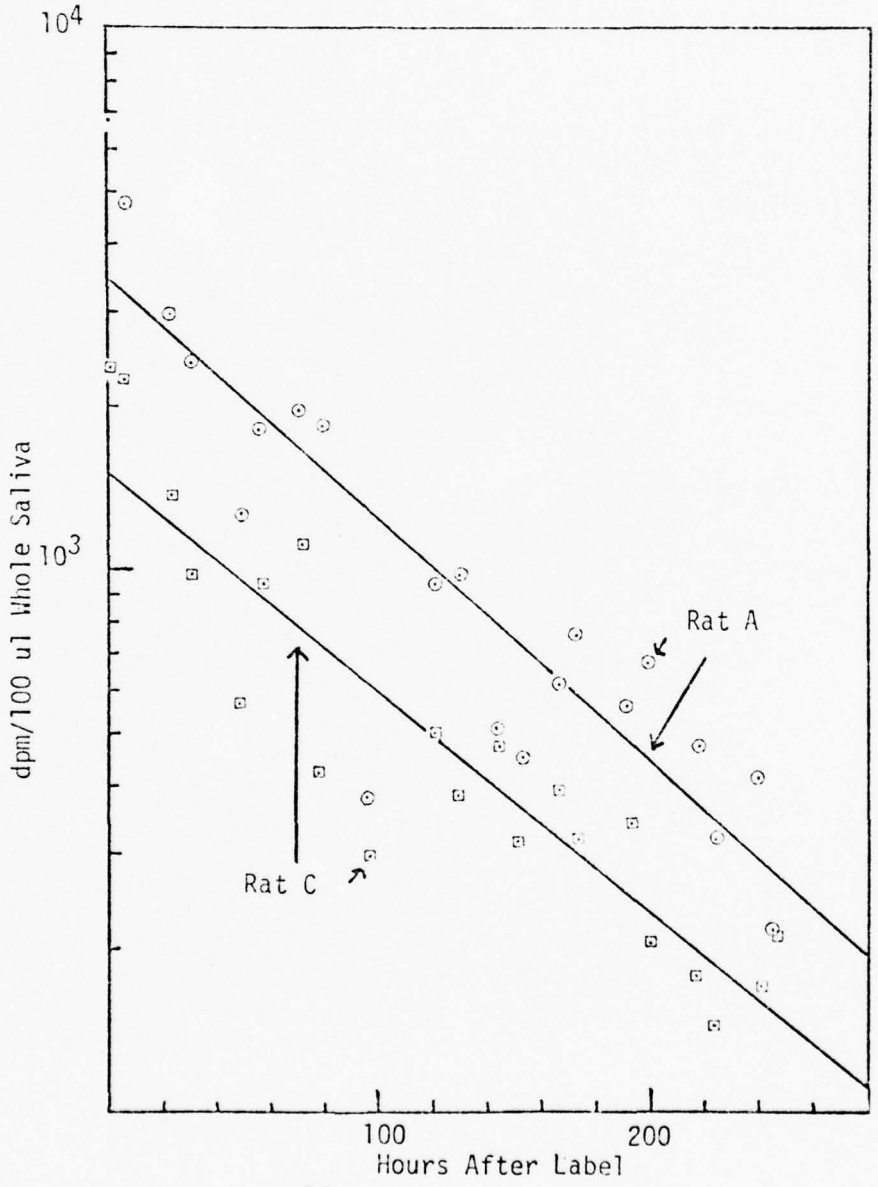


Figure 3 Excretion of ^{14}C in Saliva of Rats A and C, Each Labeled with Approximately 50 µCi/10 mg 1-^{14}C-As.

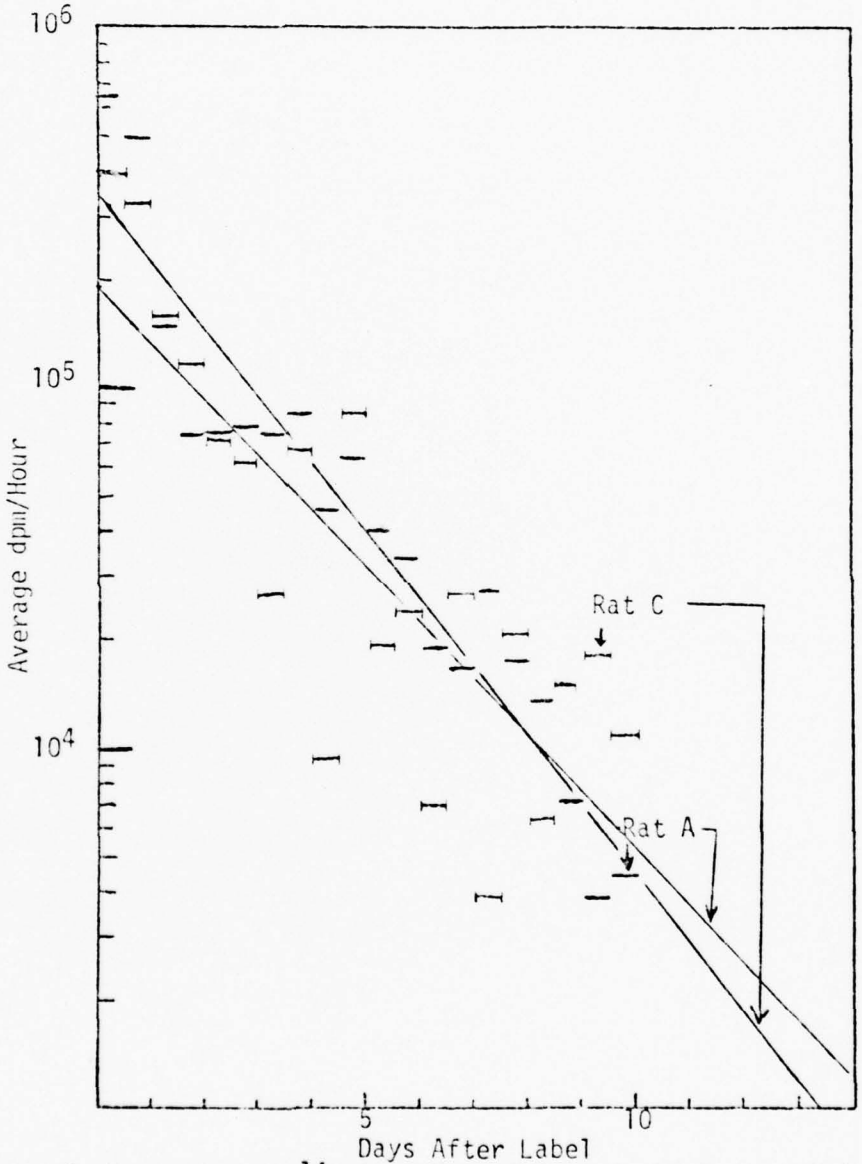


Figure 4 Excretion of ^{14}C in Urine of Rats A and C, Each Labeled with Approximately 50 μCi/10 mg 1-^{14}C-As.

TABLE I

Biological Half-Life of ^{14}C in Saliva and Urine of Rat Given 1-^{14}C-As

Rat	mg 1-^{14}C-As injected	Figure		Biological Half-Life of ^{14}C			
		Saliva	Urine	Saliva		Urine	
				Days	Hours	Days	Hours
A	10 mg	I	II	2.8	67.4	3.3	78.6
A	10 mg	III	IV	2.8	66.7	1.9	46.3
C	10 mg	III	IV	3.1	74.3	1.6	38.2
B	3 mg	I	--	2.1	51.0	---	----
A,A &C	10 mg each	---	--	Mean ($\bar{x}$) 2.9 σ^{*}=0.2	Mean ($\bar{x}$) 69.4 σ= 4.2	Mean ($\bar{x}$) 2.3 σ=0.9	Mean ($\bar{x}$) 54.4 σ=21.4

$^{*}\sigma$ is the standard deviation.

gland slowly increased its ^{14}C level, peaking at 4 hours, in mice. No measurement was done on the parotid gland, however (26).

The ^{14}C in saliva of the 1-^{14}C-As labeled rats declined logarithmically over time, as did the urinary ^{14}C. This first order process in saliva and urine was graphically analyzed by the least squares method, and the biological half-life of ^{14}C was calculated. The biological half-life of ^{14}C in rats labeled with 10 mg 1-^{14}C-As is an average of 2.9 days in saliva and 2.3 days in urine (Table I). The variability of the urine excretion data is large, so that there is no statistical difference in the observed salivary biological half-life and the biological half-life in urine. The biological half-life of ^{14}C in saliva may be a less variable measure of the overall body turnover of label than that of the excreted urinary label, which fluctuates due to the amount of urine excreted, time of day, duration of collection time and loss of urine during handling.

The salivary glands of rats, mice and guinea pigs rapidly accumulate large amounts of ^{14}C when given 1-^{14}C-As by injection. However, saliva is not a major excretory route of ^{14}C in 1-^{14}C-As labeled rats. Averaged data from three rat experiments where 10 mg of 1-^{14}C-As was injected, resulted in only 0.05% of the ^{14}C injected being excreted in saliva during a 20-30 minute saliva collection at about one hour after the final radioactive injection.

Whenever such a small percentage of the original As label is being observed, there is always the possibility that the observed label results from an artifact of the 1-^{14}C-As. This artifact could result, for instance, from the breakdown of 1-^{14}C-As even before

labeling of the rat or from the breakdown and reincorporation of ^{14}C into the body pool of another compound. If the observed ^{14}C in saliva were from a body pool other than the body pool of ascorbic acid, a biological half-life would be expected that differed from the biological half-life of As calculated from the excretion of ^{14}C in urine. Since there is no statistical difference between the biological half-life of ^{14}C in saliva and that for ^{14}C in urine of 1-^{14}C-As labeled rats, then it can be concluded that the ^{14}C observed in saliva did not result from an artifact.

Figure 3 evidences the difficulties encountered in dividing the contents of radioactive vials. Each rat should have received 10 mg of 1-^{14}C-As. However, rat A, which received the vial rinses, showed a much higher specific activity of ^{14}C in saliva than did rat C. Yet, if the specific activities for rats A and C (taken from Figure 3) are averaged, then it will be seen that the average comes very close to the specific activity of ^{14}C in saliva of rat A in Figure 1, where rat A had received the entire contents of a radioactive vial and multiple rinses.

Chapter II

INVESTIGATION OF SALIVA FOR PRESENCE OF As AND SAs

Part A: Column Chromatography of Human Saliva for As and SAs

Introduction

Experiments using ^{14}C in humans was not feasible in this laboratory, but analysis of human saliva was desired for two reasons: 1) to add to the knowledge of As metabolism in humans, 2) as a convenient source of large quantities of saliva. Liquid chromatography was chosen as a convenient method of separation because of its capacity to separate large quantities of materials. C. S. Smith (20) reported in 1976 that ion-exchange chromatography was successful in the separation of dehydroascorbic acid (dAs) and As in salivary samples. He reports that 50% of the As excreted in saliva is dAs. Using the 2,2'-dipyridyl assay (Zannoni), Smith reports that the total As level (dAs + As) is close to blood levels. Blood levels of As in humans are generally assayed by the DNPH method and are reported to be about 1 mg % (13). Feller et al. (19) reported no free As in saliva. Tolbert et al. (7) reported a C-6-As oxidation process of about 50% of the excreted urinary ^{14}C-As metabolites. These reports suggest a Zannoni assay reactive material which is not free As and may be a C-6-As oxidation derivative. A candidate for this unknown C-6 oxidized derivative was saccharoascorbic acid (SAs), which was available as a standard in this laboratory. Ascorbic

acid and SAs are both good UV absorbers. Thus, human saliva was analyzed for the possible presence of As and SAs using ion-exchange chromatography with UV monitoring of the liquid chromatographic fractions in the procedure below.

Methods

Human Saliva Collection

Paraffin stimulated saliva was collected and, if not immediately used, refrigerated at 0°C until use. Most saliva was collected from a female subject taking 2 g As tablets/day.

Column Chromatography

Whatman DE 32, a DEAE cellulose [O-(diethylaminoethyl) cellulose (formate form)], was the column support. The eluent was a buffer gradient starting with water and ending in 0.5M ammonium formate adjusted to pH 3 with 88% formic acid (buffer equivalent to 2.5M formate). A 200 ml gradient of 100 ml water and 100 ml formate buffer was used to elute a 1 cm I.D. by 30-40 cm length (small) column and a 400 ml gradient of 200 ml of each was used to elute a 1.5 cm I.D. by 75 cm length (medium) column. In all of the column work, the 2.5M formate buffer was used as the strip solution. A peristaltic pump was used to give a small column flow of 0.6-1.3 ml/minute. The UV absorbances of the column work were at 254 nm. The small column runs were done at room temperature. The medium column runs were done at 3°C.

TCA Precipitation and Centrifugation

Trichloroacetic acid (TCA) was added to a sample until a pH of approximately 3 was obtained and precipitation occurred. Usually this was accomplished by adding 2 ml of 5% w/v TCA to 5 ml of saliva, which resulted in a 1.4% w/v TCA solution. The TCA precipitated sample was then centrifuged for about 10 minutes at 13,000 RPM at 1°C in a Sorvall RC2-B refrigerated centrifuge.

Results

A DE 32 small column chromatograph of 1.0 ml human saliva (from a male who was taking no supplemental As) which had been TCA precipitated and centrifuged shows two major UV-absorbing peaks labeled PI and PII in Figure 5. A small UV peak labeled PPI (pre-peak I) is also shown. Saliva was collected from both male and female subjects. The chromatograph of the saliva from the male subject (Figure 5) resulted in 1.2 for the ratio of the areas of PI/PII and in 2.5 for that of saliva from a female subject taking 500 mg As/day. Saliva from both male and female subjects resulted in no chromatographic UV absorption position difference of PI and PII. Analysis of human saliva collected from female subjects taking 2 g As tablets/day and subjects taking 500 mg As/day showed no differences in the UV absorbance peak area or positions of PI and PII by column chromatography. 10 µg each of As and SAs applied to the column resulted in a UV absorbance of 0.0055 for As and 0.0043 for SAs (see Table II). In runs using standards only, (i.e., no saliva), the UV absorbance for an As standard was observed at approximately

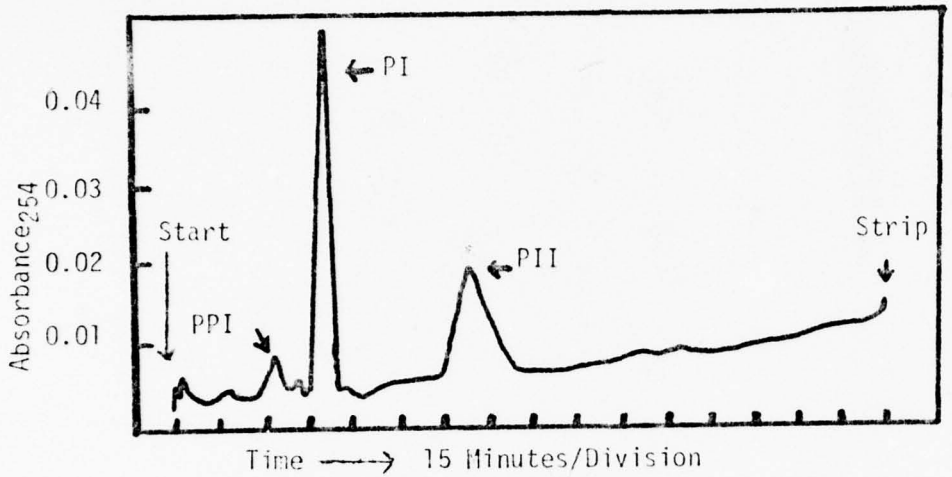


Figure 5 DE 32 Column Chromatograph of TCA Precipitated-Centrifuged Saliva from Human Male. The average flow-rate was 0.8 ml/minute. PPI, PI, and PII eluted at 24 ml, 39 ml, and 85 ml, respectively.

TABLE II

Comparison of Estimated UV Absorbance of the As Peak On the Small (1 x 30-40 cm) DEAE Cellulose Column With the Ability to Measure the Absorbance at Various Ranges

Full Scale Recorder Range	Smallest Division on Abs Scale of Recorder Chart Paper	Applied µg As	Estimated Small Column Peak Maximum of As UV Abs
0.020	0.040×10^{-2}	1	0.055×10^{-2}
0.050	0.10×10^{-2}	2	0.11×10^{-2}
0.10	0.20×10^{-2}	5	0.28×10^{-2}
0.20	0.40×10^{-2}	10*	0.55×10^{-2}
0.50	1.0×10^{-2}	20	1.1×10^{-2}
1.0	2.0×10^{-2}	40	2.2×10^{-2}
2.0	4.0×10^{-2}	80	4.4×10^{-2}

*Actual measurement of 10 µg As at range 0.050 gave an absorbance of 0.55 -- other absorbance values are calculated from this measured value assuming a direct proportionality between the amount of substance applied and the maximum UV peak absorbance. The maximum UV peak absorbance of 10 µg SAs was measured at 0.0043 on this column.

the same fraction as that for PI, and the UV absorbance for an SAs standard was observed at approximately the same fraction as that for PII. The positions of As, SAs, As-2-SO_4, SAs-2-SO_4, and As-2-PO_4 on a small column chromatograph are indicated in Figure 6.

A DEAE 32 (formate form) small (1 x 30-40 cm) column chromatograph of 1.0 ml of a centrifuged mixture of 9.0 ml of saliva from a human female taking 2 g As/day, 1 ml water solution containing 0.10 mg As and 0.10 mg SAs, and 4 ml of 5% w/v TCA is shown in Figure 6. The eluent was a gradient beginning with H_2O and ending with a 2.5M formate buffer (pH 3.0), which was the strip solution. The 1 ml of mixture applied to the column (Figure 6) contained 0.64 ml saliva and 7.1 µg each of As and SAs. The expected UV absorbance (calculated from Table II) of 7.1 µg As is 0.0039 and of 7.1 µg As is 0.0031. Since no new UV-absorbing peaks result from adding As and SAs to saliva and they would be clearly visible by themselves on this chromatograph (Figure 6), then it must be concluded that the standards coincide with PI and PII.

Discussion

This preliminary investigation of saliva using a UV monitored liquid chromatograph indicated that it was possible for both As and SAs to be present, but with no other major UV-absorbing substances. This did not account for the report of the presence of dAs (20) in saliva, however, because dAs does not significantly absorb at 254 nm. Also, no UV-absorbing peaks were observed for As-2-PO_4, As-2-SO_4, and SAs-2-SO_4 (Figure 6). The two major absorbing peaks

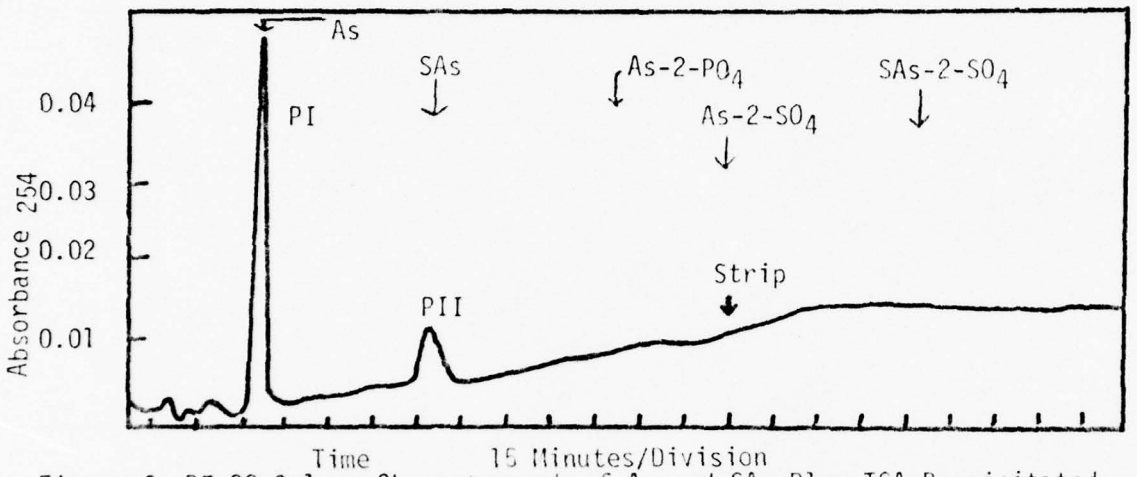


Figure 6 DE 32 Column Chromotograph of As and SAs Plus TCA Precipitated-Centrifuged Human Saliva; Position of Standards. The average flow-rate was 1 ml/minute. PI and PII eluted at 39 ml and 98 ml, respectively.

in human saliva were observed in both sexes, but not in the same proportion. Human saliva of a female taking orally 2 g As/day in the form of tablets did not show enhanced UV absorption peak areas per ml of saliva over a female taking 500 mg supplemental As. No UV-absorbing peak positions were affected, nor were new UV absorbing peaks observed

Part B: TLC and Paper Chromatography of Human Saliva for As and SAs

Introduction

The possibility of the presence of As in saliva has been shown using column chromatography in the previous part of this chapter. In addition, there is also the possibility that SAs may be present in appreciable amounts. Further experiments to investigate the possible presence of As and SAs were necessary; thin-layer chromatography (TLC) and paper chromatography were chosen as the analytical tools, as outlined below.

Methods

Ultrafiltration

The saliva was diluted before filtering with an equal volume of either water or 2.5M, pH 3.0 formate buffer (described under column chromatography). The Amicon ultrafilter (UF) was employed with the Diaflo UM 05 filter (retentivity > 500 MW). All UF work was done in a cold room at 3°C with N_2 gas.

Thin-Layer Chromatography

System I

Solvent

n-Butanol: Acetic Acid: Carbon Tetrachloride (70:15:15).

Solid Support

Sigma Silica Gel H mixed 1g:2g H_2O and coated 1 mm thick on glass plates. Plates were air dryed and then oven-dried at 80°C for several hours immediately prior to use.

System II

Solvent

n-Butanol: Acetic Acid: H_2O (3:2:2).

Solid Support

Polygram Cel 300 UV_{254} Pre-coated Cellulose plates. Layer: 0.1 mm made by Machercy-Nagel & Co. - Düran.

Paper Chromatography

Whatman filter paper — Solvent system was n-Butanol: Acetic Acid: H_2O (3:2:2).

Descending Eluent.

Column Chromatography (previously described).

Results

Human saliva was collected as previously described. Two 7.1 ml samples of human saliva were TCA precipitated, centrifuged, and chromatographed on the small DEAE column. The UV-absorbing fractions previously designated as PPI, PI and PII were collected

and evaporated with N_2 to dryness at room temperature. The total UV absorbance was equivalent to the absorbance of 0.426 mg As for PI and 0.102 mg SAs for PII. All fractions were reconstituted with water and about 80% of PI, 50% of PII, 100% of PPI, As, SAs and a mixture of As and SAs were applied to a TLC plate. The TLC was accomplished using System I. Quantitative standards of As and SAs had been done using System I. TLC plates were examined under UV light (254 nm), sprayed with 0.5 mM dichloroindophenol (DCIP) followed by reaction with iodine vapor. The minimum detection limits of this system used were 0.01 mg As (detected by iodine) and 0.024 mg SAs (detected by iodine). A 0.5 mM DCIP spray detected a minimum of 0.05 mg of As and SAs. No As or SAs was detected in PI or PII on this TLC plate (Figure 7). Both applied peaks should have been visible by iodine and DCIP spraying if As or SAs had been present in the quantities indicated by the peaks' UV absorbances.

PI and PII fractions were lyophilized to dryness from a medium column run of 800 ml of human saliva which had been ultrafiltered (UF) with an equal volume of formate buffer. A small column run of some of this lyophilized PII showed two UV-absorbing peaks. The lyophilized product PII consisted of a mixture of fluffy white material (mp 187 ± 7°C) and a yellow cream material (mp > 300°C). A TLC of the two solid components of lyophilized PII (Figure 8) was done on TLC System II along with standards of As, SAs, As-2-SO_4, As-2-PO_4, and 2,3-di-O-methyl-As. The Rf values of the standards and unknowns on this TLC plate (Figure 8) are measured from the leading edge of each spot. Two spots were visualized by iodine

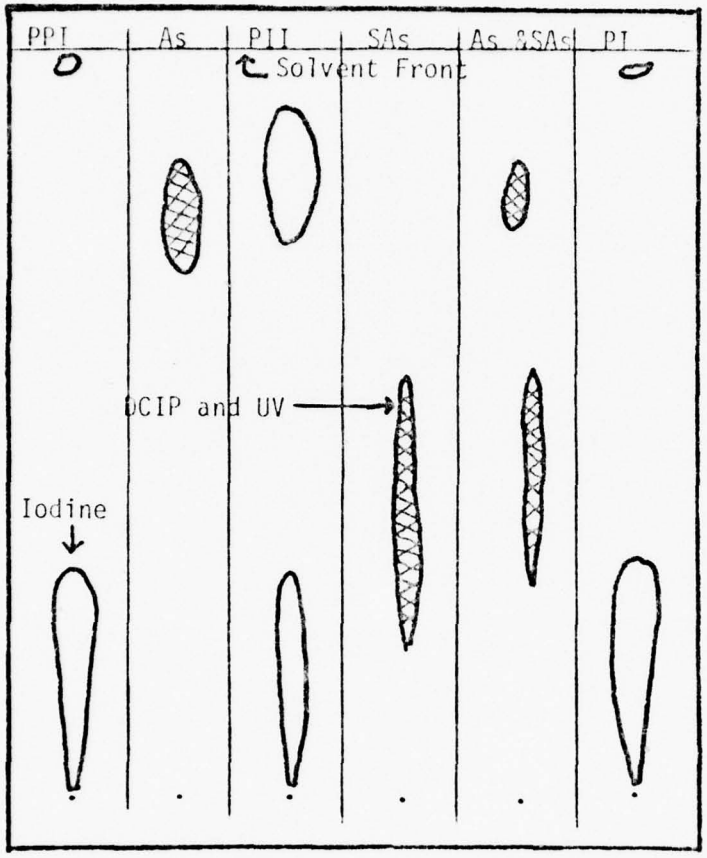


Figure 7 TLC (System I) of the DE 32 Column Fractions PPI, PI, and PII from Human Saliva.

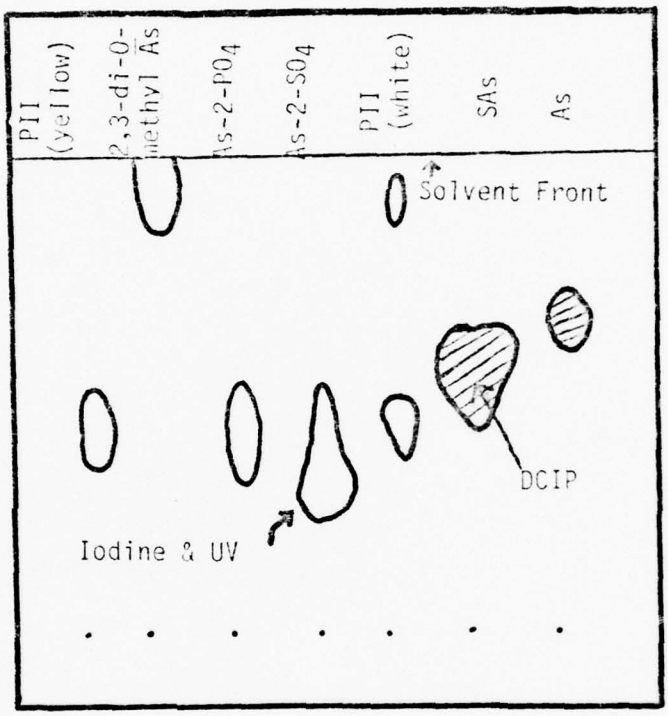


Figure 8 TLC (System II) of the DE 32 Column PII Fractions from Human Saliva.

reaction for the white PII solid with Rf's of 0.49 and 0.97. The yellow PII solid produced one iodine reactive spot at an Rf of 0.49. The As, SAs, As-2-SO_4, As-2-PO_4, and 2,3-di-O-methyl-As standards had Rf's of 0.73, 0.66, 0.49, 0.50, and 1.0, respectively. As-2-PO_4 and As-2-SO_4 have about the same Rf as one of the spots present in both PII solids, but the PII fractions are not close to the fractions where these standards elute on the DE 32 column (Figure 6). The PII spot with an Rf of 0.97 is close to that for 2,3-di-O-methyl-As. Paper chromatography of each of these PII solid components (Figure 9) shows Rf's (measured from the leading edge of each spot) for PII solids differing from As and each other but close to the expected Rf of SAs in this paper chromatography system.

Discussion

The preliminary analysis of human saliva using ion-exchange chromatography monitored by UV absorption at 254 nm indicated the possible presence of As and SAs. UV absorbances, marked PI and PII on the UV recording of the column run, coincided with the UV absorbances of the added As and SAs standards, respectively.

Examination of PI and PII of human saliva by TLC and paper chromatography did not show the presence of As or SAs, although there was some material present with Rf's close to the expected Rf of SAs in the paper chromatograph. Also, there was no evidence of the presence of SAs-2-SO_4 in PII using TLC and no evidence of As-2-SO_4, As-2-PO_4 and SAs-2-SO_4 in PI or PII using column chromatography. There was some substance present in PII that had a similar

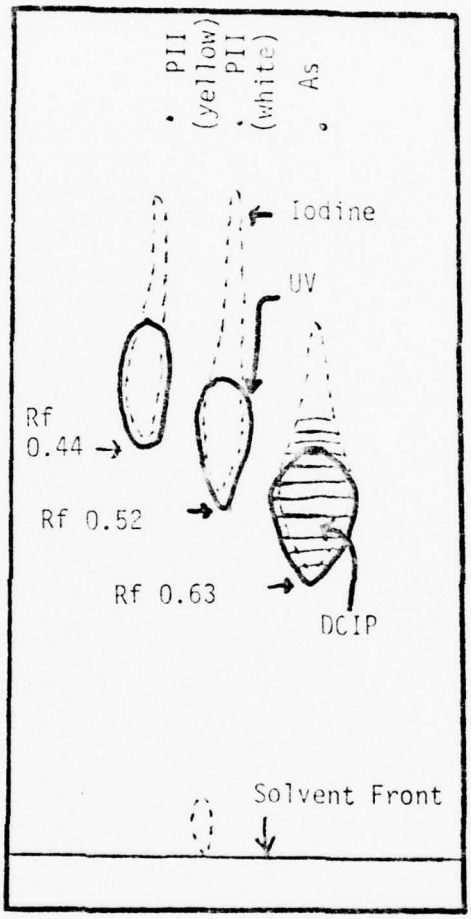


Figure 9 Decending Paper Chromatography of the DE 32 Column PII Fraction from Human Saliva.

Rf to 2,3-di-O-methyl-As in System II of the TLC and in the paper chromatograph.

Part C: Co-Chromatography of As and SAs with Saliva from 1-^{14}C-As Labeled Rats

Introduction

In Part B of this chapter, it was reported that no evidence of As in human saliva was obtained from TLC and paper chromatography. A column chromatographic confirmation of the absence of As in rat saliva was also desired. The close Rf's of PII solids with SAs in the paper chromatography data required further investigation of the absence or presence of SAs. In the following experiments, a radioactive label was employed for the detection of As metabolites in the liquid chromatography fractions, since there had been interference between the UV-absorbing peaks of compounds in saliva and UV-absorbing peaks of standards.

Methods

Column Chromatography (previously described).

Results

0.240 ml of radioactive rat saliva was spiked with 0.60 mg SAs, TCA precipitated, centrifuged, and applied to the small column (Figure 10). The major radioactive peak appeared at fraction 14 in this run, while the UV absorbance for SAs clearly peaked early in fraction 13. There was a small overlap in UV absorbance attributed

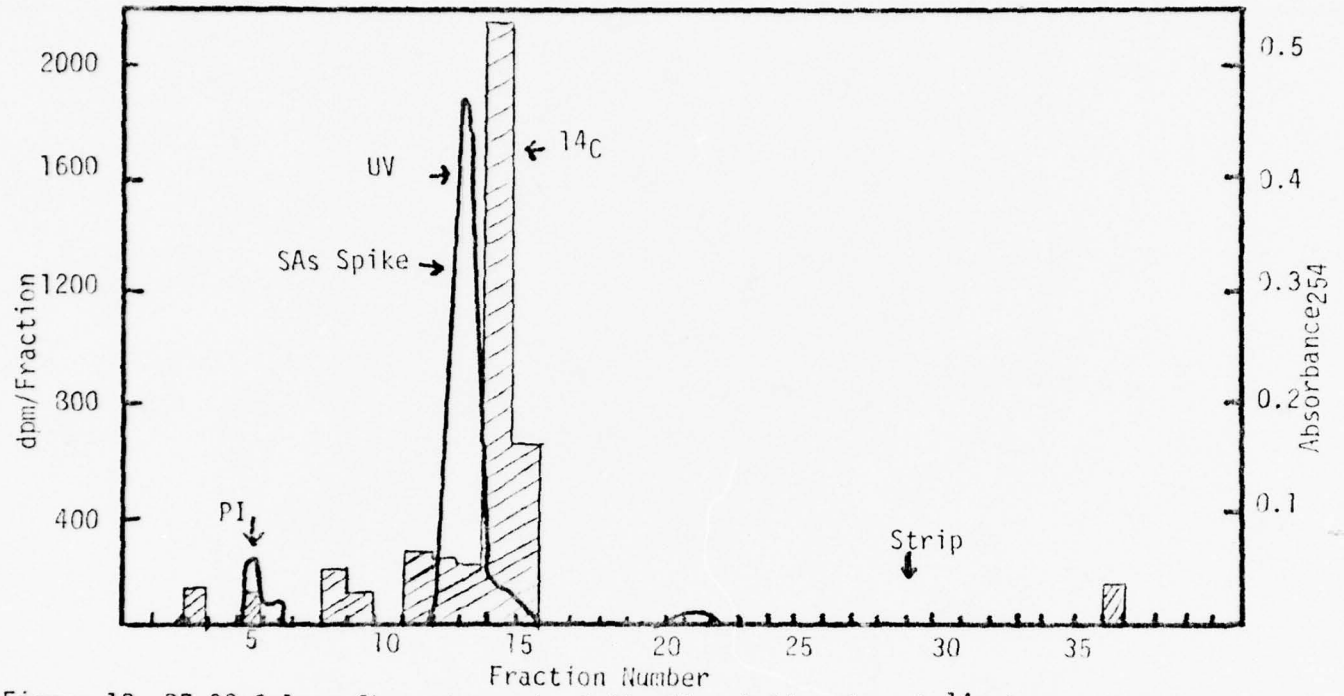


Figure 10 DE 32 Column Chromatograph of SAs Plus Saliva from 1-^{14}C-As Labeled Rat. The average volume per fraction was 7.1 ml. PI, SAs, and the major ^{14}C peak eluted at 33 ml, 79 ml, and 92 ml, respectively.

to the SAs spike and the major radioactive peak. If the sum of the counts of fractions 14 and 15 are assigned a value of 100%, then no other radioactive peaks shown are greater than 10% of this major peak. Also, this major peak constitutes greater than 50% of the combined radioactivity of all fractions. Figure 10 shows some amount of UV absorbance corresponding to the major radioactive peak. UV-absorbing PI also had a trace amount of radioactivity associated with it in this column chromatograph.

0.400 ml of radioactive rat saliva was spiked with 0.050 mg As, TCA precipitated, centrifuged, and applied to the small column (Figure 11). Particular attention was paid to radioactivity balance in this column chromatography run, and no loss to the column support was observed. In this run, an air bubble obscured the detection of UV absorbance at fraction 7, where a trace of radioactivity was observed. Nevertheless, this run clearly showed no radioactivity associated with the UV absorbance as the As spike. It was also noted that the UV absorbance of PII corresponds to the major radioactive peak in this column chromatograph.

Discussion

Column work done with saliva from 1-^{14}C-As labeled rats confirmed both the results reported by Feller et al. (19) and the nonradioactive TLC and paper chromatography results described previously, by showing that no detectable amount of ascorbic acid is present in saliva (Figure 11). Also, column chromotagraphy of

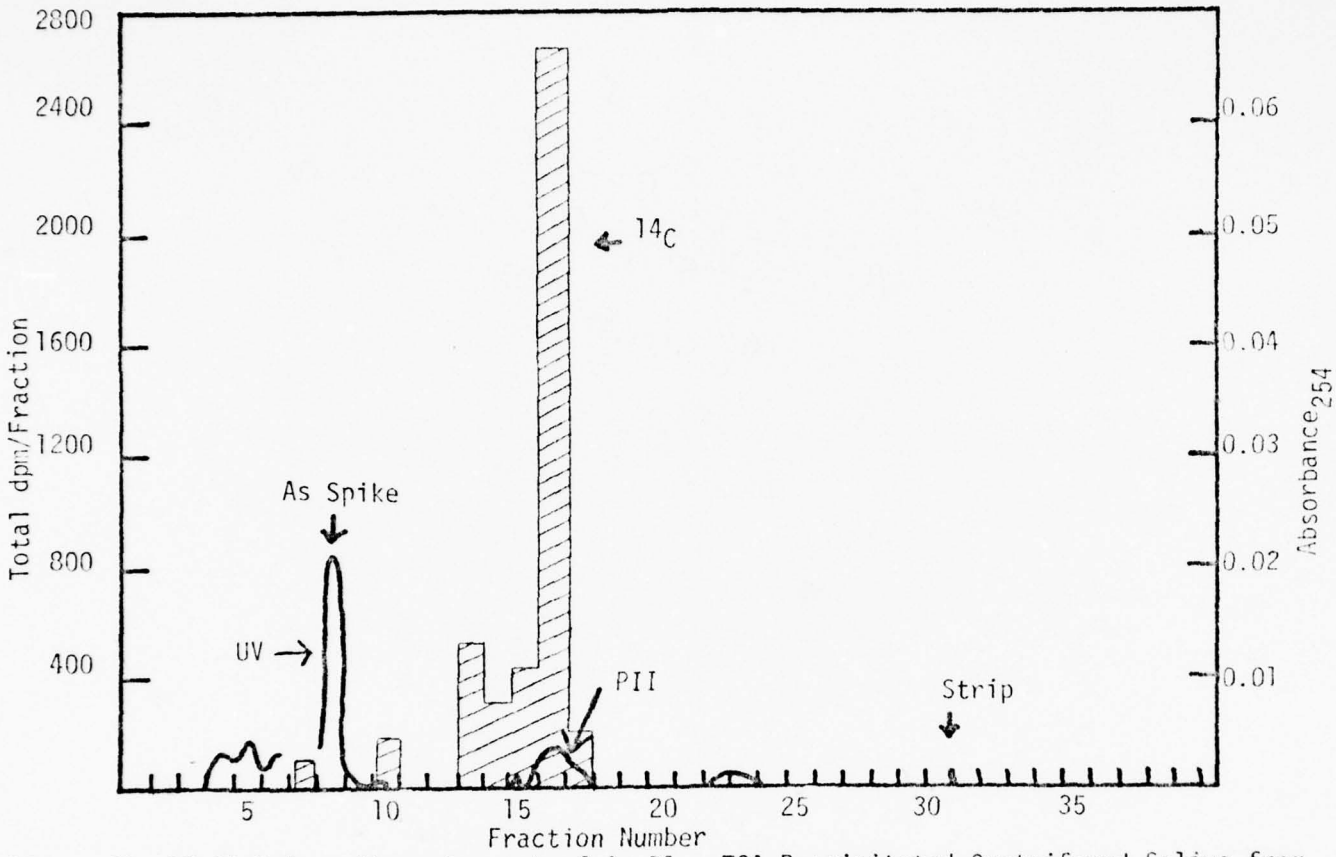


Figure 11 DE 32 Column Chromatograph of As Plus TCA Precipitated-Centrifuged Saliva from 1-^{14}C-As Labeled Rat. The average volume of fractions 1-4 was 4.9 ml and of fractions 5-40 was 7.5 ml. The As spike eluted at 33 ml and both PII and the major radioactive peak eluted at 94 ml.

radioactive saliva spiked with SAs confirmed that the major metabolite was not SAs (Figure 10). Furthermore, only one major 1-^{14}C-As metabolite was shown to be present in radioactive rat saliva using column chromatography, and this metabolite appears to be a fairly strong acid.

Chapter III

INVESTIGATION OF THE 1-^{14}C-As METABOLITE FOR UV ABSORBANCE

Part A: UV Absorption of 1-^{14}C-As Metabolite from Rat: Acid Pretreatment Followed by Column Chromatography

Introduction

Ascorbic acid and As derivatives with an intact enediol-lactone ring are all excellent UV absorbers having molar extinction coefficients on the order of 10^4. Monitoring liquid chromatography by UV absorption is desirable because of its convenience. The intact enediol-lactone ring is the chemical structure responsible for the reactivity of As and SAs in the 2,2'-dipyridyl and DCIP tests and leads to the DNPH color reaction. This is also the structure that makes As and SAs good UV absorbers. All of these facts lead to the assumption that the observed column UV absorbances of saliva coincident with the 1-^{14}C-As metabolite were due to the metabolite's UV absorption. Significantly, a few column UV analyses did not coincide perfectly with the radioactivity and are described in the following results.

Methods

Column Chromatography (previously described).

Results

Three small column runs showed close, but imperfect coincidence of the major radioactive peak with UV-absorbing PII. One

typical example of this is shown in Figure 12. Figure 12 shows the major radioactive peak maximizing at fraction 16 and the UV absorbance of PII maximizing at the beginning of fraction 17. All three of these column runs had the same saliva pre-column treatment, that is, the addition of TCA to precipitate proteins and centrifugation.

Discussion

Throughout most of the column work, the major radioactive peak appeared to be closely associated with some major UV absorbance (Figure 12). Further column work made the coincidence of UV absorbance with the radioactivity suspect. The accuracy achieved in correlating the fractions collected with the UV absorbance recorded does not permit attributing non-coincidence to measurement errors.

Part B: UV Absorption of 1-^{14}C-As Metabolite from Rat: Neutral pH Pretreatment Followed by Column Chromatography

Introduction

Errors in the marking of column fractions on the UV recorder chart paper could not account for the separation of the UV-absorbing peak and the radioactive peak by a whole fraction. However, the separation of peaks was small, and a greater separation was desired. Better separation of the UV-absorbing peaks may be obtained by using a larger column eluted with a greater total volume of gradient and by using a different pretreatment of the saliva. The results of these modified column chromatography procedures are given below.

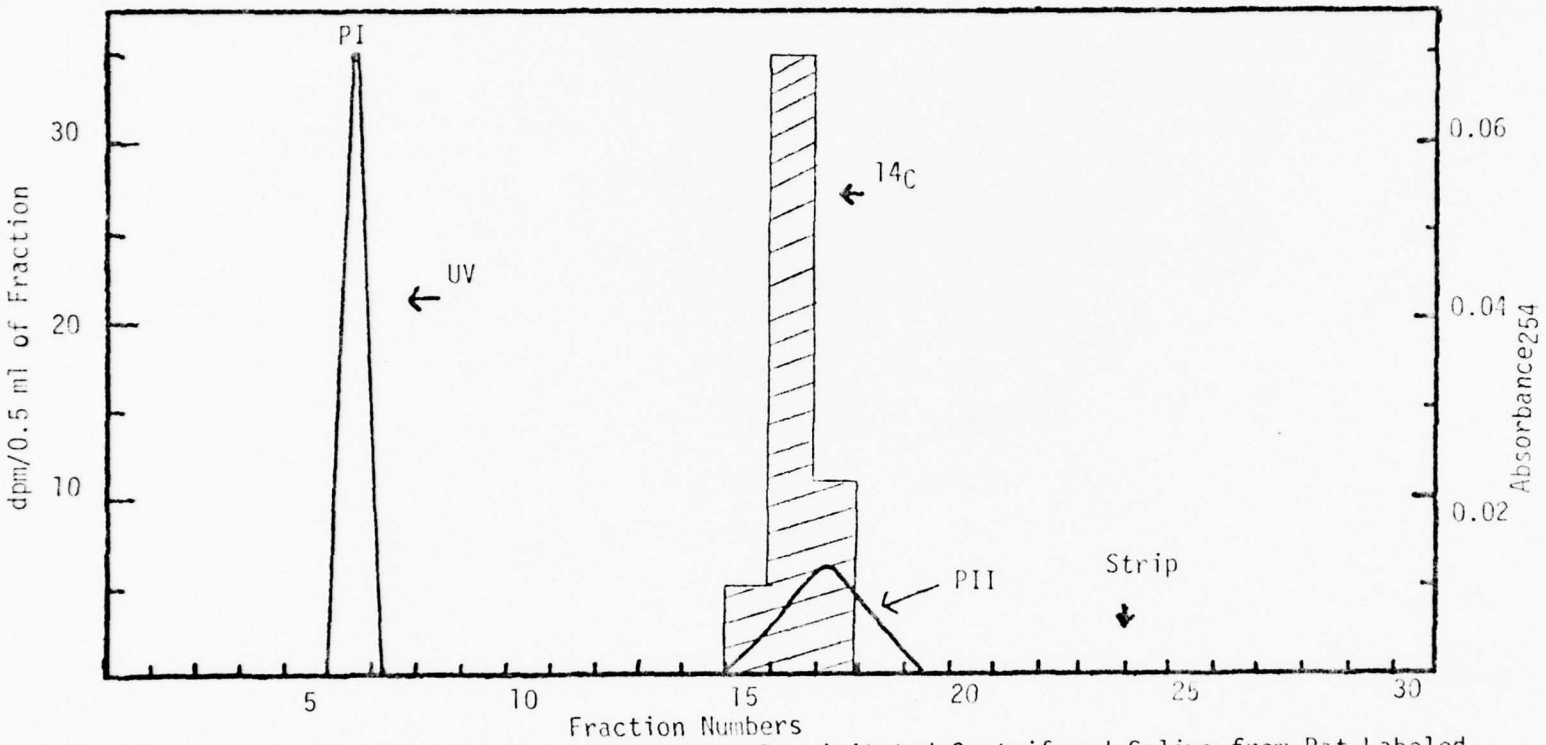


Figure 12 DE 32 Column Chromatograph of TCA Precipitated-Centrifuged Saliva from Rat Labeled with 1-^{14}C-As. The average volume per fraction was 8.7 ml. PI, PII, and the major ^{14}C peak eluted at 31 ml, 100 ml, and 93 ml, respectively.

Methods

Column Chromatograph (previously described).

Ultrafiltration (previously described).

Results

The results of a medium column run of 3.2 ml of radioactive rat saliva and 800 ml human saliva which had been ultrafiltered in formate buffer and lyophilized then combined and applied to the column is shown in Figure 13. Standards of As and SAs previously chromatographed on the medium column had appeared at fractions 60 and 90, respectively, by monitoring UV absorption. Fractions 75-84 encompassed the major radioactive peak which did not coincide with the UV-absorbing PII. Similarly, the maximum UV absorbance of PI corresponds to only a trace of radioactivity. A different radioactivity distribution is observed on this run, the major peak containing only twice as many counts for the fractions 75-84 as for the fractions 36-40 and 130-133. This change in distribution may be due to breakdown of the labeled compound. The medium column run took about 24 hours from the start of the 400 ml gradient to the end of the gradient (beginning of the strip), whereas the peristaltic pump application of the 200 ml gradient to the small column took only about three hours. In view of the observed distribution change from small to medium columns, the longer time evidently allowed some breakdown of labeled compounds, though the medium column was refrigerated.

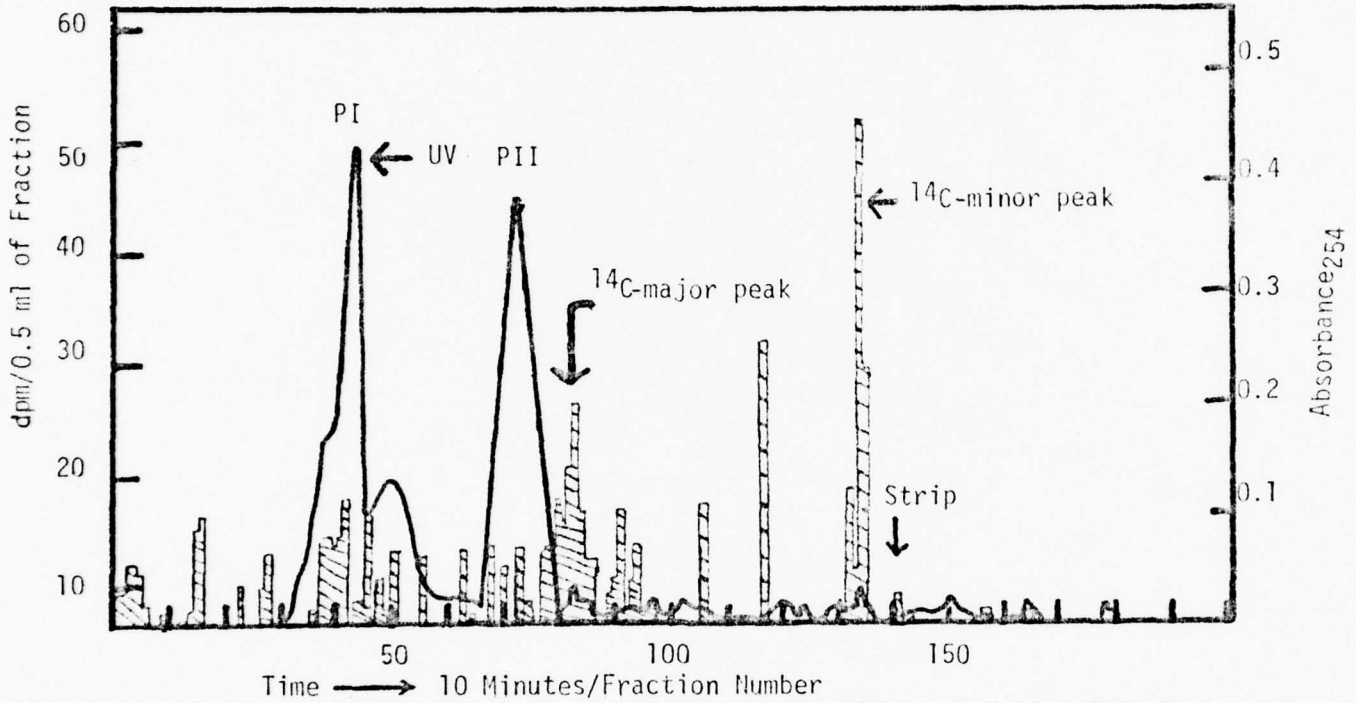


Figure 13 DE 32 Column Chromatograph of Acidified, Ultrafiltered Saliva from Human and 1-^{14}C-As Labeled Rat. The average volume of fractions 1-2 was 10 ml and of fraction 3-200 was 2.8 ml. PI, PII, the major ^{14}C, and the minor ^{14}C peaks eluted at 138ml, 218 ml, 247 ml, and 389 ml, respectively.

Pre-column treatment of saliva by diluting with water and ultrafiltering resulted in complete separation of the radioactive peak and the UV-absorbing peaks (Figure 14). In this small column run, a mechanical failure resulted in fraction 20 not being collected. However, the counts contained in the fraction were estimated by subtracting the total radioactivity eluted from the amount applied, a reasonable calculation since no loss of radioactivity to the column support had been observed in previous column chromatography using the small column. This estimate is indicated in Figure 14. The estimated peak absorbances for different amounts of As applied, based on the actual peak maximum absorbance of As when run on the small column, are shown in Table II. If reported values of about 1 µg As/ml saliva as determined by the DNPH assay are used as a basis of estimation, then 0.6 µg As should have been present in this run (Figure 14). This amount of As would not be measurable by UV absorption if it were applied to the small column (Table II).

Discussion

A change of pre-column treatment from acidic conditions to a water dilution with subsequent ultrafiltration eliminated the coincidence of observed UV absorption with the major radioactive peak (Figure 14) of a column chromatograph of radioactive rat saliva.

The nature of the UV-absorbing weak acid components of saliva is not known. Preliminary UV absorption measurements of PII fractions show a λ_m of 240 nm at pH 3 from one column run of human saliva and 241.5 nm at pH 3 from one column run of rat saliva. 800 ml of saliva

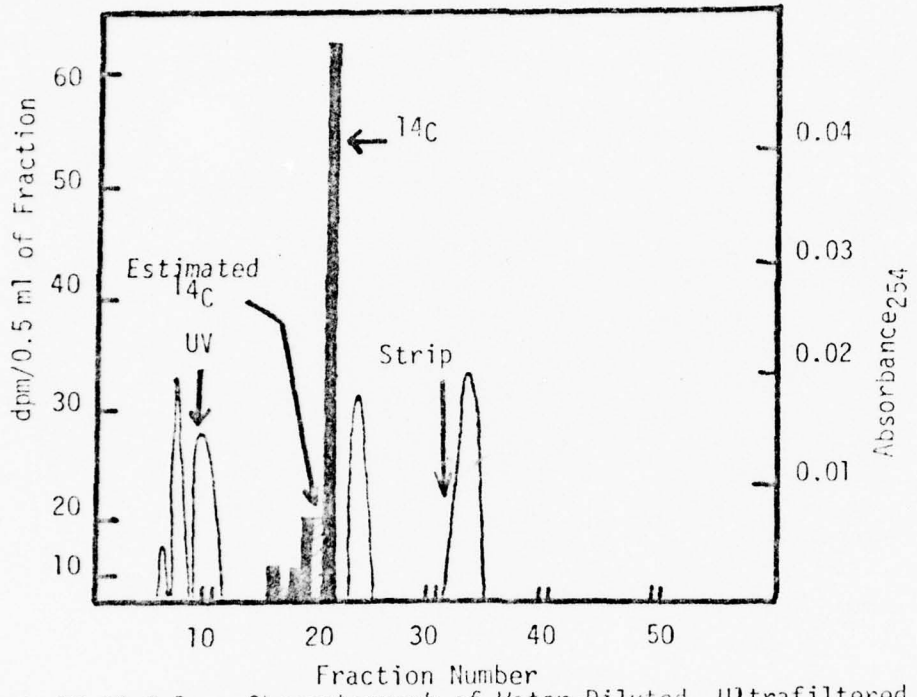


Figure 14 DE 32 Column Chromatograph of Water-Diluted, Ultrafiltered Saliva from 1-^{14}C-As Labeled Rat. The average volume per fraction was 5.8 ml. The UV-absorbing peaks measured at fractions 6, 8, 10, 23, and 33 eluted at 26 ml, 33 ml, 50 ml, 132 ml, and 190 ml. The major ^{14}C peak eluted at 114 ml.

which had been ultrafiltered with formate buffer were chromatographed, and UV-absorbing PI and PII each had λ_m's of about 243 nm and 281 nm. The absorbance at 281 nm may be a derivative of tryptophan or tyrosine. Mass spectra of lyophilized material from UV-absorbing PII showed only low molecular weight fragments.

The elution of the 1-^{14}C-As metabolite of rat saliva using ion-exchange chromatography is characteristic of a fairly strong acid, possibly an α-hydroxycarboxylic acid. The fact that the compound elutes after ascorbic acid using a column gradient eluent from water to acid indicates that it is a stronger acid than As. Side chain oxidation or degradation of As is capable of producing this change.

Chapter IV

INTERFERENCE IN ASCORBIC ACID ASSAYS

Part A: Possible Ascorbic Acid Metabolite Interference in Ascorbic Acid Assays

Introduction

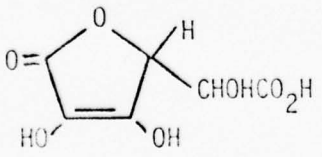
The most common procedures for determining ascorbic acid (As) in biological tissues are based on the presence of an intact enediol-lactone ring of As. Results from such experimental procedures on biological samples are usually reported as As. However, compounds such as derivatives of As with the enediol-lactone ring intact or derivatives of As with acid labile substituents may be able to interfere with these As assay methods.

The dinitrophenylhydrazine (DNPH) assay of ascorbic acid is a standard method used to measure As concentrations in biological tissues. Three DNPH methods commonly employed are the Schaffert-Kingsley (27), Roe-Kuether (28), and Bessey-Lowry (29) procedures, which differ essentially only in the time and temperature of the osazone color development. Roe (3) has already emphasized avoidance of high temperatures during the coupling reaction. Baker et al. (6), using the modified DNPH procedure of Roe-Kuether, have reported that one known metabolite, As-2-SO_4, can give the same color reaction as ascorbic acid. When the As-2-SO_4/DNPH assay mixture was heated for 15 minutes at boiling water temperature (95°C in Denver), it reacted equally as well as As. As-2-SO_4, assayed at 60°C for one hour using

this DNPH method, resulted in 50% of the As-2-SO_4 being converted to the colored osazone, as compared with 100% of the As converted under these same conditions. At 37°C for one hour, no DNPH color development was observed for As-2-SO_4. Interference by As-2-SO_4 is particularly important in urine, where As-2-SO_4 excretion has been demonstrated (30).

Preliminary results have been reported recently that approximately 45% of the urinary As metabolites in rats and monkeys have undergone side chain oxidation at the C-6 position, presumably leaving the enediol-lactone ring intact (7, 31, 32). L-threo-saccharoascorbic acid (SAs) is a 6-carbon oxidation derivative of As with an intact enediol ring. SAs is one possibility for a side chain oxidation metabolite of As with an intact enediol-lactone ring. SAs was tested for interference in the following three As assay methods: 2,2'-dipyridyl; 2,4-dinitrophenylhydrazine (DNPH); and 2,6-dichloroindophenol (DCIP).

L-threo-Hex-2-enaro-1,4-lactone[IUPAC] or, commonly, L-threo-saccharoascorbic acid (SAs) was tested using the three As assay methods below. The SAs was prepared by air oxidation of the C-6



L-threo-saccharoascorbic acid

carbon of L-ascorbate-2-sulfate, using a Pt/C catalyst. The SAs-2-sulfate formed was then deblocked by acid hydrolysis and recrystallized

to greater than 99% purity. Details of this procedure have been published (8).

Methods

2,2'-Dipyridyl Assay

The 2,2'-dipyridyl colorimetric test followed the procedure of Zannoni, et al. (4). The enediol ring of As reduces Fe^{III} to Fe^{II}. The Fe^{II} is complexed with 2,2'-dipyridyl to produce a red color which is measured at 525 nm with a spectrophotometer; the color development is linear with the amount of As.

DNPH Assay

The 2,4-dinitrophenylhydrazine (DNPH) colorimetric As assay used was the Schaffert-Kingsley procedure (27), modified by Baker (33). Trichloroacetic acid is added to the sample; the sample should be centrifuged, if a precipitate is observed. The modification is substitution of cupric ion in place of charcoal (Norit) as the oxidizing agent of As. One milliliter of DTC mixture [10.0 ml (2 gm DNPH in 100 ml 9N H_2SO_4), 1.0 ml (0.32% $CuSO_4$), and 1.0 ml (1.0% thiourea)] is added to each milliliter of sample. The substitution of DTC for Norit eliminates the filtration step and, in some cases, a centrifugation step necessary for the removal of the Norit. DNPH couples with dehydroascorbic acid to form a red-colored osazone. The color is read at 515 nm with a spectrophotometer. The development of the red color of the osazone is directly proportional to the amount of As reacted.

DCIP Titration

The 2,6-dichloroindophenol (DCIP) As assay method used was a titration procedure. A 0.477 mM DCIP aqueous solution was used to titrate both As and SAs. Each sample aliquot was buffered at pH 7 with a monobasic potassium phosphate-sodium hydroxide buffer so that a blue endpoint could be visualized. Ascorbic acid reduces DCIP on an equimolar basis.

Results

The 2,2'-dipyridyl experimental procedure assayed SAs on an equimolar basis with As, as reported in Table III. The DNPH test assayed SAs on an equimolar basis when color development took place at 56° for 1 hour, as shown in Table IV. When the DNPH assay was run at 37° for 1 hour, the absorbance vs. moles reacted curve was not linear; the ratio of the absorbances for equimolar amounts of SAs/As was 1.34. When the DNPH assay was run at 37° for 3 hours, this ratio was close to one. Thus, the SAs appears to react more rapidly with DNPH than does As. The DCIP titration procedure assayed SAs on an equimolar basis with As, as shown in Table V. The molar extinction coefficient and λ_m of SAs was measured in a monobasic potassium phosphate-sodium hydroxide buffer at pH 7.0 and found to be 1.40×10^4 and 267 nm, respectively.

Discussion

The data reported in this paper show that SAs, a possible metabolite of As, can interfere in the As assays used in this study

TABLE III

2,2'-Dipyridyl Assay of As and SAs

Absorbances at 525 nm are recorded of duplicate runs I and II for the molar amount shown of As and SAs. Absorbances of the blanks have been averaged (0.008) and subtracted.

Sample Amount nmoles	As (Absorbance) I	As (Absorbance) II	SAs (Absorbance) I	SAs (Absorbance) II
7.10	0.027	0.019	0.029	0.021
14.2	0.046	0.038	0.040	0.032
28.4	0.083	0.075	0.083	0.075
42.6	0.118	0.110	0.107	0.099
56.8	0.150	0.142	0.155	0.147

TABLE IV

2,4-Dinitrophenylhydrazine Assay of As and SAs

Absorbances at 515 nm are recorded of duplicate runs I and II for the molar amounts shown of As and SAs. Color was developed at 56° for 1 hour. Absorbances of the blanks have been averaged (0.010) and subtracted.

Sample Amount x 10^{-8} moles	As (Absorbance) I	As (Absorbance) II	SAs (Absorbance) I	SAs (Absorbance) II
5.68	0.193	0.189	0.184	0.186
11.4	0.377	0.364	0.353	0.367
17.1	0.507	0.516	0.520	0.529
22.8	0.674	0.661	0.669	0.683
28.5	0.838	0.834	0.864	0.843

TABLE V

2,6-Dichloroindophenol Titration of As and SAs

Based on the As standard, the molarity of the DCIP solution is 0.477 mM for this study. The average ml of DCIP for four separate titrations of each substance is reported.

Sample	ml DCIP (Ave. of 4 titrations)	$\frac{\text{ml DCIP}}{\text{mole}} \times 10^6$	$\frac{\text{moles DCIP}}{\text{mole}}$
4.00 moles As	8.39 ± 0.22[a]	2.10 ± 0.06	1
5.30 moles SAs	10.98 ± 0.23	2.07 ± 0.04	0.99 ± 0.07

[a]Standard deviation of 4 values.

on an equimolar basis. This result confirms the expectation that side chain oxidation derivatives of As with an intact enediol-lactone ring interfere with these As assay methods. Derivatives of As in which the enediol-lactone ring is unsubstituted should respond to all three procedures. If the ring is substituted, the response would depend on the lability of the derivative. Therefore, As-2-sulfate cannot be assayed by the DCIP or the 2,2'-dipyridyl procedures, but can be assayed by the DNPH procedure if done at elevated temperatures (6). As-2-phosphate is more readily hydrolyzed than As-2-sulfate and should cause more interference.

Since side chain oxidation products of As have been reported to be a significant portion of As metabolites from at least one biological source, then it is possible that a number of analytical results of As assays on blood, urine and tissue have significant errors from metabolite interference.

Part B: DNPH Reactive Material of Human Saliva

Introduction

Odumosu and Wilson (16) reported that centrifuged human saliva had one sixth the DNPH reactive material of uncentrifuged human saliva. Acidification of the saliva with TCA and filtration to remove the Norit also removes some DNPH reactive material. Neither centrifugation nor filtration should remove free As or a small metabolite of As.

DNPH assays of human blood, urine, and saliva revealed no correlation of the levels of DNPH reactive material in human saliva with any of the other two tissues (13, 17). Perhaps the sought-for correlation of the DNPH reactive material in saliva with that in other tissues does not exist. In this section, data are presented showing that the DNPH assay is not measuring As or the major 1-^{14}C-As metabolite in saliva, but, instead, is measuring interfering substances.

Methods

DNPH Assay (previously described).

Ultrafiltration (previously described).

Column Chromatography (previously described).

Results

4658 dpm/100 µl was the average value for rat saliva collected approximately one hour after the radioactive injection (Figure 3), where 50 µCi/10 mg 1-^{14}C-As was administered to each rat. The dpm/ml for this value is 46580 dpm/ml of rat saliva. The moles of 1-^{14}C-As metabolites per ml of rat saliva may be calculated by assuming that:

1) the tissue level of As in the rat is comparable to that in man and that the rat's body pool can be estimated as follows:

$$\frac{2 \times 10^3 \text{ mg As (human body pool)}}{70 \times 10^3 \text{ g human}} \times 350 \text{ g rat} = 10 \text{ mg As (rat body pool)};$$

2) the 1-^{14}C-As has equilibrated with the As in the body pool; and

3) the molar specific activity of the radioactivity is not different for As and its salivary metabolites.

The number of moles of As metabolite per milliliter of rat saliva is:

$$\frac{4.658 \times 10^{4} \text{ dpm}}{1 \text{ ml rat saliva}} \times \frac{1 \text{ } \mu\text{Ci}}{2.2 \times 10^{6} \text{ dpm}} \times \frac{10 \text{ mg As injected} + 10 \text{ mg As (rat body pool)}}{50 \text{ } \mu\text{Ci total injected}}$$

$$\times \frac{1 \text{ mole As}}{0.176 \times 10^{6} \text{ mg As}} = 4.8 \times 10^{-8} \text{ moles As metabolites/ml rat saliva}$$

If equal color development from DNPH reactions with As and with As metabolites represents equal moles of those substances present, then for each ml of whole (uncentrifuged) rat saliva, there is about 4.5×10^{-8} moles of As metabolites (Table VI). Approximately 4×10^{-8} moles of As metabolites/ml also applies to the equivalent As molar levels in uncentrifuged human saliva as measured by DNPH assay. TCA precipitated-centrifuged rat saliva yielded about 2/5 as much DNPH reactive material as uncentrifuged material, and an even larger drop in DNPH reactive material was observed for TCA precipitated-centrifuged human saliva (Table VI). It must be noted that 40-45% of the radioactivity of 1-^{14}C-As labeled rat saliva was also lost upon precipitation with TCA and centrifugation or ultrafiltration with an equal volume of 2.5M formate buffer (pH 3). By contrast, ultrafiltration with an equal volume of water yielded no significant loss of radioactivity in rat saliva, yet the DNPH material of water-diluted, ultrafiltered human saliva dropped to much less than the equivalent of 0.1 µg As/ml (6×10^{-10} mole As/ml) level.

TABLE VI

Specific Activity of 1-^{14}C-As Metabolites in Saliva and the Level of DNPH Reactive Material in Saliva

Source of Saliva Sample*		Treatment of Saliva Sample	Calculated Moles of 1-^{14}C-As Metabolites/ml Saliva	Equivalent As Moles of DNPH Reactive Material/ml Saliva (Color developed 1 hour at 56°C)
Rat	Injected Subcutaneously	TCA Precipitated and Centrifuged	Assumed Body Pools of 10 mg As in Rat and 2 g As in Human	
A	10.0 mg As	Yes	----------	1.99×10^{-8}
A	10.0 mg As	No	5.02×10^{-8}	----------
A	10 mg As	No	7.09×10^{-8}	----------
A	None	No	----------	4.20×10^{-8}
B	None	No	----------	4.71×10^{-8}
B	3 mg As	No	6.64×10^{-8}	----------
A&B	None	No	----------	6.70×10^{-8} (by 2,2'-dipyridyl method)
C	10 mg As	No	2.54×10^{-8}	----------
C	1.0 mg As	No	0.994×10^{-8}	----------
# of Human Samples	Administered Orally			
1	2 g As/day	Yes	----------	0.45×10^{-8} (uncertainty high)
1	2 g As/day	Yes	----------	0.79×10^{-8}
1	2 g As/day	Yes	----------	0.77×10^{-8}
12	Trace As	No	1.8×10^{-8}	----------
1	2 g As/day	No	----------	(cloudy) by eye about 4.2×10^{-8}

*The radioactive human saliva was generously supplied by Dr. Anders Kallner at Huddinge University Hospital, Huddinge, Sweden.

Figure 15 shows a small (1 x 30-40 cm) column run of 80 ml of human saliva and 3.3 ml of saliva from a rat labeled with 1-^{14}C-As. The saliva was ultrafiltered with an equal amount of water at 3°C and lyophilized to dryness. The lyophilized saliva was reconstituted in double-distilled water and applied to the column. UV absorbance at 254 nm was measured. Radioactivity was determined by counting 0.5 ml of each 12 ml fraction. The remaining fractions were lyophilized to dryness before a DNPH assay was run on the remaining portion of each fraction. The DNPH reactive levels obtained were adjusted to include the missing 0.5 ml aliquots used for counting the radioactivity. As previously mentioned, all controls (10 ml aliquots) for the DNPH reaction showed that much less than 0.1 μg As/ml (6×10^{-10} mole As/ml) DNPH reactive material was present after the human saliva was ultrafiltered in water. Of the three human saliva controls, two controls were lyophilized before the DNPH assay was started, and one was concentrated to approximately 2.5 ml by evaporation with N_2 and assayed. No further tests were carried out on the material coming off the column which was DNPH reactive, so the identity of this material is not known. It is known, however, that this material is not from contaminated column material, because two of the control chromatographs and the Figure 15 chromatograph were accomplished using fresh column material; all of these showed easily detectable levels of DNPH reactive material in the fractions collected, when essentially no DNPH reactive material was applied.

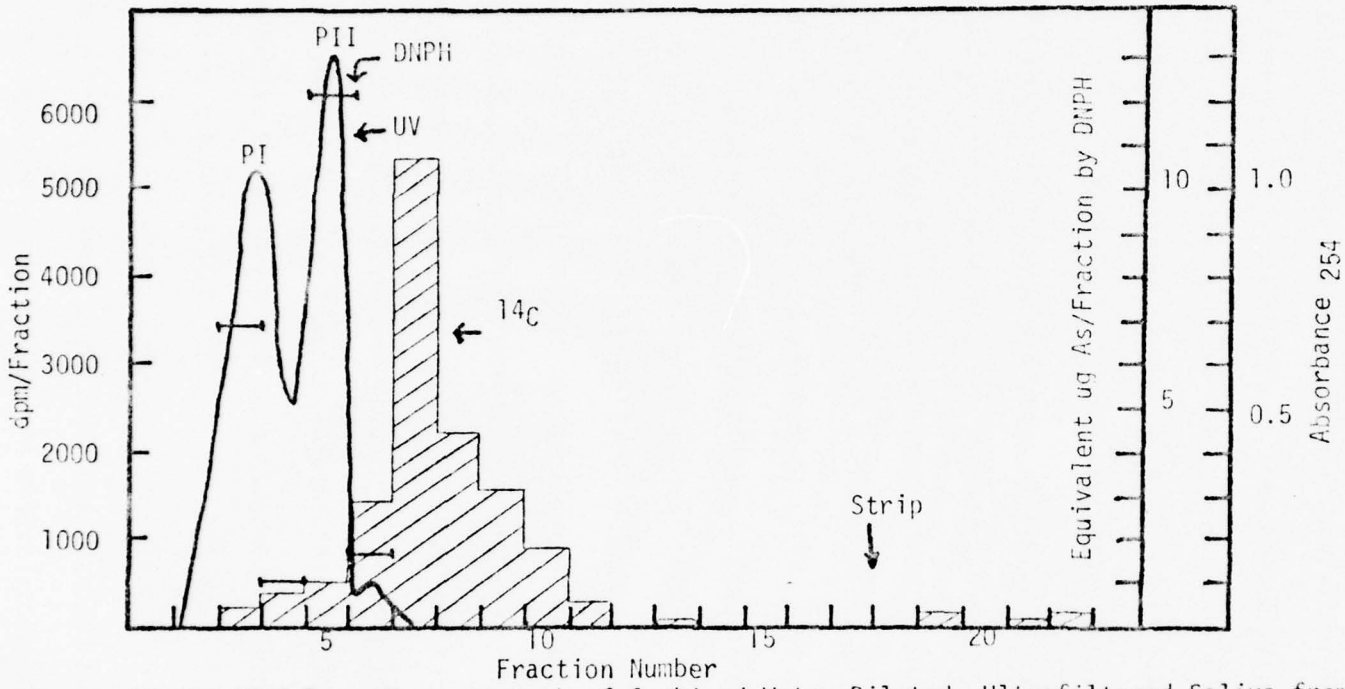


Figure 15 DE 32 Column Chromatograph of Combined Water-Diluted, Ultrafiltered Saliva from Human and 1-^{14}C-As Labeled Rat. The average volume of fractions 1-4 was 10 ml and of fractions 5-22 was 12 ml. PI, PII and the major ^{14}C peak eluted at 26 ml, 46 ml, and 70 ml, respectively.

Discussion

TCA precipitation and centrifugation or ultrafiltration in formate buffer resulted in a 40-45% loss of the radioactivity of saliva. DNPH reacted aliquots of human saliva which were TCA precipitated and centrifuged were two fifths as DNPH reactive as the saliva which was TCA precipitated but not centrifuged. The nonconcurrance of DNPH reactivity losses with radioactivity losses suggested that the DNPH assay was not measuring the radioactive metabolites. This was confirmed when the DNPH reactive material of 10 ml of human saliva (after ultrafiltration with water) was equivalent to much less than 0.1 μg As/ml (6×10^{-10} mole As/ml) whole saliva. By contrast, no significant radioactivity of the rat saliva was lost in the same UF/water step. A mixture of human saliva and radioactive rat saliva was ultrafiltered in water. The ultrafiltered mixture was applied to the small DEAE column. The DNPH test was carried out on the fractions from this column run, and some DNPH reactive material was observed for the UV-absorbing fractions. However, no DNPH reactive material was observed in the major radioactive peak (Figure 15). This appearance of DNPH reactive material was not due to column contamination, because fresh column material was used for the chromatography in Figure 15 and in two other separate control runs.

Since the ultrafiltration membrane's retentivity is greater than 500 MW, then the As metabolite must be less than 500 MW. Based on the column chromatography results, the As metabolite appears to be a fairly strong acid. It is apparently not DNPH reactive. The 3.3 ml

of radioactive rat saliva applied to the column run (Figure 15), alone, should have given a detectable level of DNPH reactive material if all of the DNPH reactive material were concentrated in the fractions of the major radioactive peak (fractions 7 and 8); however, no DNPH reactive material was observed for the major radioactive peak.

The observation of Odumosu and Wilson (16) that TCA precipitated-centrifuged human saliva had a fraction of the DNPH reactive material of whole human saliva was confirmed, as shown in Table VI. Similarly treated radioactive rat saliva resulted in the loss of only 40-45% of the radioactivity. Further analysis using a water dilution of human saliva which was ultrafiltered resulted in essentially complete loss of DNPH reactive material, whereas ultrafiltration of water-diluted radioactive rat saliva resulted in no significant loss of radioactivity. The ultrafilter membrane having a retentivity of greater than 500 MW should not have removed any free ascorbic acid nor any of its known metabolites. Therefore, there is no justification for reporting DNPH reactive material in saliva as ascorbic acid or as a metabolite of As. Some candidates for the DNPH reactive material of whole saliva are carbohydrates resulting from hydrolysis of the polysaccharide portion of mucin. Hydrolysis of the polysaccharide is likely, because of the strong acid conditions of the DNPH analysis. The losses of DNPH reactive material observed in acidified saliva could be due to the partial TCA precipitation of the glycoprotein-mucin and its removal by centrifugation or filtration.

Chapter V

CALCULATIONS

Part A: Calculation of the Expected UV Absorbance for the 1-^{14}C-As Metabolite

Introduction

Previous experiments show that the major 1-^{14}C-As metabolite in saliva does not coincide with any significant amount of UV absorbance using a DEAE cellulose column chromatograph. The presence or absence of UV absorption of the metabolite is of interest. If an intact enediol-lactone ring is present, it should have a molar extinction coefficient around 10^4 and a λ_m at 240-260 nm. It is possible to calculate an expected UV absorbance of an As metabolite in saliva with an intact enediol-lactone structure by using radioactivity levels of the ascorbate metabolite; this calculation is done below.

Calculation

An estimate can be made of the expected UV absorption of the 1-^{14}C-As salivary metabolite by making a number of assumptions:

1) that the metabolite is UV-absorbing, and that its molar extinction coefficient is on the same order as that for ascorbic acid (about 10^4);

2) that tissue levels of As in the rat are comparable to that in man, and that the rat's body pool can be estimated as follows:

$$\frac{2 \times 10^3 \text{ mg As (human body pool)}}{70 \times 10^3 \text{ g human}} \times 350 \text{ g rat} = 10 \text{ mg As rat body pool;}$$

3) that man makes the 1-^{14}C-As metabolite found in rat saliva;

4) that the amount of metabolite/ml rat saliva appearing in the major radioactive column peak is equal to the amount of metabolite/ml human saliva that would be present in corresponding column fractions;

5) that a reasonable estimate of the amount of metabolite present can be obtained by using the maximal peak absorbance of an As standard as directly proportional to the amount of As applied when the same column is used (Table II);

6) that the molar specific radioactivities for As and its metabolite do not differ;

7) that the 1-^{14}C-As is equilibrated with the As body pool; and

8) that the column chromatographic peak shape of the 1-^{14}C-As metabolite is comparable to that for As.

With these assumptions, the following calculation may be made for the column chromatograph of Figure 15:

$$\frac{1 \text{ mole As}}{0.176 \times 10^{6} \text{ mg As}} \times \frac{7585 \text{ dpm (fractions 7+8)}}{3.3 \text{ ml of rat saliva applied}} \times \frac{\text{10 mg As rat body pool + 10.0 mg As injected}}{\text{50 } \mu\text{Ci total injected}}$$

$$\times \frac{1 \ \mu\text{Ci}}{2.2 \times 10^{6} \text{ dpm}} = 2.4 \times 10^{-9} \text{ moles As metabolite/ml whole saliva}$$

Assumption (5) allows peak heights and not peak areas to be used as an estimate of the absorbance one might expect for the column chromatographic peak of the 1-^{14}C-As metabolite. The observed peak absorbance of the 0.01 mg As applied to the small DEAE column is 0.0055 (from Table II). In addition to the 3.3 ml of radioactive rat saliva, 80 ml of human saliva was lyophilized to dryness,

re-dissolved in a small volume and applied to the small column, the chromatograph of which is shown in Figure 15. In this chromatograph, the expected absorbance of the 1-^{14}C-As salivary metabolite would be:

$$\frac{0.0055 \text{ abs}}{0.01 \text{ mg As}} \times \frac{0.176 \times 10^{6} \text{ mg As}}{1 \text{ mole As}} \times \frac{2.4 \times 10^{-9} \text{ mole As metabolite}}{1 \text{ ml whole saliva}}$$

$$\times 80 \text{ ml human saliva applied} = 0.019 \text{ abs expected for 1-}^{14}\text{C-As salivary metabolite}$$

At the full scale recorder range of 2.0 abs, as indicated on Figure 15, an absorbance of 0.02 is about one half of the smallest chart paper division, which is detectable. No UV absorbance was observed for the radioactive peak (Figure 15).

Discussion

No UV absorbance was observed for the 1-^{14}C-As metabolite of saliva under these experimental conditions. However, no conclusion may be drawn as to whether the metabolite is UV-absorbing because the calculated expected absorbance of the 1-^{14}C-As metabolite of saliva was within the background variation. Thus, it remains an open question whether the enediol-lactone ring remains intact in the major 1-^{14}C-As metabolite of rat saliva.

Part B: Calculation of the Moles of 1-^{14}C-As Metabolites Per Milliliter of Rat and Human Saliva and Column Chromatography of Radioactive Human Saliva

Introduction

Based on radioactivity calculations done by Baker et al. (34) to determine the size of the As pool in man, there are about 2 grams As in the human body pool. A specific activity for the metabolite in saliva can be calculated by assuming that the molar specific activities of the metabolites and As are equal.

Methods

A human experiment investigating the pharmacokinetics of As metabolism was conducted by Dr. Anders Kallner at Huddinge University Hospital, Huddinge, Sweden, who generously agreed to supply all of the radioactive human saliva referred to below. The methods of administration of 1-^{14}C-As and saliva collection described below were carried out by Dr. Kallner.

Administration of 1-^{14}C-As to Human Subjects

Human subjects were given a trace amount of 1-^{14}C-As totaling 20 μCi orally.

Radioactive Human Saliva Collection

Saliva was stimulated with a quick rinse of citric acid. Saliva collections took place at the third, fourth, and fifth hours for 20 minute periods. Bottles of saliva were flushed with nitrogen and stored at below 20°C. The collection date was November 8, 1976.

Dr. Kallner then shipped the collected saliva on dry ice to this lab. Four bottles were thawed January 25, 1977 and counted. The remaining 8 bottles were thawed May 4, 1978 and counted, ultrafiltered with water, and lyophilized. Then a column chromatograph of this lyophilized material was done.

Results

Specific radioactivity calculations (a sample of this calculation is given below) for man given a trace of As and for rat given 1 mg As result in 1.8×10^{-8} mole As metabolite/ml human saliva and about 0.994×10^{-8} mole metabolite/ml rat saliva (Table VI). The rat dpm/ml saliva is calculated from data given in Chapter I. The human dpm/ml saliva data is listed in Table VII).

Sample Calculation

Assuming that: 1) the molar specific radioactivities for As and its metabolite do not differ, and

2) the 1-^{14}C-As is equilibrated with the As body pool,

then

$$\frac{1\ \mu Ci}{2.2 \times 10^{6}\ dpm} \times \frac{1\ mole\ As}{0.176 \times 10^{6}\ mg\ As} \times \frac{\#\ of\ dpm}{1\ ml\ whole\ saliva}$$

(Rat: 1 hour after label Human: Mean of 12 measurements at the 3,4, and 5th hours after label)

$$\times \frac{\#\ of\ mg\ As\ in\ body\ pool + \#\ of\ mg\ As\ administered}{total\ \#\ of\ \mu Ci\ administered} = \#\ of\ moles\ As\ metabolites/ml\ whole\ saliva.$$

TABLE VII

Specific Activity of ^{14}C in the Saliva of Human Subjects Given 20 μCi of 1-^{14}C-As Orally* on 11/8/76

Hours After Label Administered That Saliva Was Collected	Dietary Intake of Human Subject	dpm/ml Saliva** Refrigerated After Thawing on 1/25/77 and Recounted on 5/4/78 (Blank Subtracted)	dpm/ml Saliva** at Date of Original Thawing (Blank Subtracted)
3	E 30 mg		37 ± 9% (5/4/78)
4	E 30 mg		64 ± 9% (5/4/78)
5	E 30 mg		34 ± 9% (5/4/78)
3	N 60 mg	43.2 ± 9%	69 ± 3% (1/25/77)
4	N 60 mg	18.2 ± 10%	50 ± 4% (1/25/77)
5	N 60 mg	26.3 ± 9%	81 ± 3% (1/25/77)
3	S 90 mg		38 ± 9% (5/4/78)
4	S 90 mg		150 ± 9% (5/4/78)
5	S 90 mg	18.5 ± 10%	49 ± 4% (1/25/77)
3	T 180 mg		197 ± 8% (5/4/78)
4	T 180 mg		53 ± 9% (5/4/78)
5	T 180 mg		24 ± 10% (5/4/78)

Mean ($\bar{x}$) and standard deviation(σ) of 12 measurements 69 σ = 50.

*The radioactive human saliva was generously supplied by Dr. Anders Kallner at Huddinge University Hospital, Huddinge, Sweden.

**One milliliter of each saliva sample was counted as described under "Counting of Radioactivity" in this thesis.

The column chromatograph shown in Figure 16 is of water-diluted, ultrafiltered saliva from humans administered 1-^{14}C-As orally. The radioactive human saliva using 1-^{14}C-As labeled subjects revealed a single major radioactive peak using the small DEAE column (procedure as previously described). Figure 16 shows the elution position of the minor 1-^{14}C-As metabolite of human saliva to be about the same as that for the 1-^{14}C-As major metabolite of rat saliva and that for the major human metabolite to be earlier.

Discussion

The amount of 1-^{14}C-As metabolites excreted in rat saliva (collected at one hour after labeling) is about 0.05% of the total label injected. The amount of ^{14}C excreted in human saliva (calculated from the twelve-sample average shown in Table VII) is roughly 0.02% of the total ^{14}C administered. From these results, it may be concluded that saliva is not a major excretory route for the 1-^{14}C-As label.

The column chromatograph of saliva from human subjects who had been administered 1-^{14}C-As orally (Figure 16), showed a single major radioactive peak. This radioactive peak elutes before and the minor radioactive peak elutes at about the expected position of the major 1-^{14}C-As metabolite had rat saliva been used. It cannot be concluded, however, from the single column run of Figure 16 whether any of the 1-^{14}C-As metabolites in human saliva is the same as those in rat saliva.

The PII UV absorbance was minimal in this chromatograph of

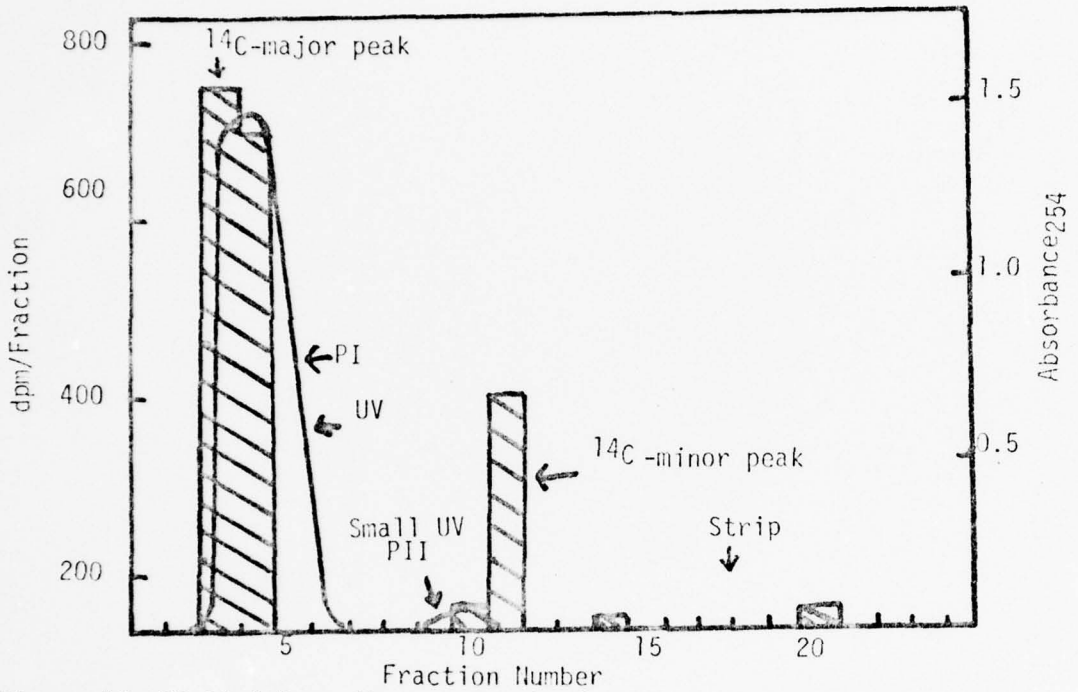


Figure 16 DE 32 Column Chromatograph of Combined Water-Diluted, Ultrafiltered Saliva from Humans Orally Administered with 1-^{14}C-As. The average volume of fractions 1-4 was 11 ml and of fractions 5-24 was 13 ml. PI, PII, the major ^{14}C, and the minor ^{14}C peaks eluted at 34 ml, 111 ml, 32 ml, and 130 ml, respectively.

human saliva (Figure 16). This decrease in PII absorbance in human saliva which had been frozen and stored has been observed several times for non-radioactive saliva samples. Therefore, it may be that the loss of PII absorbance in relationship to PI absorbance is a consequence of storing the frozen radioactive samples for prolonged periods of time.

Bacterial growth may have altered the ^{14}C metabolites of As so that Figure 16 does not reflect the human metabolites but rather bacterial or decomposition products. Further experiments using fresh radioactive saliva from humans are needed.

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Part II

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Purification and Kinetic and Physical Characterization of Bovine Ascorbate-2-sulfate Sulfohydrolase†

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ABSTRACT: Ascorbate-2-sulfate sulfohydrolase (ascorbate sulfatase) has been purified 70 000-fold to homogeneity from bovine liver. The purification procedure consists of pH fractionation, ammonium sulfate fractionation, hydroxylapatite column chromatography, Sephadex A-50 column chromatography, and preparative ultracentrifugation. Gel electrophoresis and sieving columns show that the aggregation state of the enzyme is pH dependent. Arylsulfatase and ascorbate sulfatase activities of the homogeneous enzyme show the same banding pattern on gel electrophoresis. Gel electrophoresis in the presence of sodium dodecyl sulfate and 8 M urea shows that the enzyme is comprised of two protein species of 59 000 and 52 000 molecular weight. Both of these bands stain positively for glycoprotein. The enzyme also binds to concanavalin A-Sepharose. The most powerful inhibitor of ascorbate-2-sulfate sulfohydrolase is ascorbate 2-phosphate, followed by inorganic phosphate and the nucleotide triphosphates. Ascorbate 2-sulfate hydrolysis is competitively inhibited by ascorbate 2-phosphate, Na_2HPO_4, and *p*-nitrocatechol sulfate with K_i values of 0.5 μM, 3.3 μM, and 0.1 mM. The K_m for ascorbate 2-sulfate changes from 1.3 mM to 2.9 mM upon freezing and thawing the enzyme and then to 0.5 mM upon removal of an inactive protein. Partially purified ascorbate-2-sulfate sulfohydrolase is activated by $(NH_4)_2SO_4$, while the highly purified enzyme is competitively inhibited by sulfate with a K_i of 0.04 mM. The pH optimum of partially purified ascorbate-2-sulfate sulfohydrolase is 5.4; pure enzyme, 4.8. The K_m and V_{max} values for isoascorbate 2-sulfate hydrolysis are lower than values for ascorbate 2-sulfate hydrolysis. Nitrocatechol sulfate hydrolysis by ascorbate-2-sulfate sulfohydrolase is competitively inhibited by ascorbate 2-phosphate and ascorbate 2-sulfate with K_i values of 1.8 μM and 3.8 mM, respectively. The apparent K_m for nitrocatechol sulfate hydrolysis is 0.5 mM. Both ascorbate 2-sulfate and nitrocatechol sulfate hydrolyses are substrate inhibited. It is suggested that the enzyme may have multiple unequivalent binding sites for these substrates.

Ascorbate 2-sulfate was discovered in brine shrimp cysts in 1969 (Mead and Finamore, 1969). Soon after its discovery improved procedures were developed for synthesizing ascorbate 2-sulfate salts and for quantitating it from biological tissues (March, 1972; Tolbert et al., 1975). Ascorbate 2-sulfate appears to be a ubiquitous substance in animals found in the urine or tissue of every species tested, including man (Mead and Finamore, 1969; March, 1972; Halver et al., 1975; Baker et al., 1971, 1975). The wide distribution of ascorbate 2-sulfate in animals suggests that this compound may play a role in ascorbic acid biochemistry. Ascorbate 2-sulfate is as effective as ascorbic acid in relieving scurvy in trout (Halver et al., 1975). Ascorbate 2-sulfate appears to be converted to ascorbic acid in vivo and ascorbic acid is converted to ascorbate 2-sulfate in primates (Baker et al., 1975). Therefore the enzymatic hydrolysis of ascorbate 2-sulfate to ascorbic acid should be a nutritionally important step in certain biological systems. Experiments were undertaken to isolate and characterize the enzyme activity which carries out the hydrolysis of ascorbate 2-sulfate to ascorbate.

Preliminary results have shown that a crude bovine liver arylsulfatase A (EC 3.1.6.1) preparation hydrolyzes ascorbate 2-sulfate to ascorbate (Bullen, 1972). This enzyme activity was named ascorbate-2-sulfate sulfohydrolase (ascorbate sulfatase). It has been shown that ascorbate sulfatase activity is present in many tissues from several species of animals (Knight, 1974). More recently, the partial copurification and characterization of ascorbate sulfatase and arylsulfatase from the liver of a marine gastropod, *Charonia lampas*, have been reported (Hatanaka et al., 1975a). The two enzymes responded differently to certain inhibitors (Hatanaka et al., 1975b,c). Ascorbate 2-sulfate is also a substrate for pure arylsulfatase A (Roy, 1975). Partial purification from bovine liver, using ammonium sulfate reverse phase column chromatography, has shown that arylsulfatase A and ascorbate sulfatase activities copurify (Carlson, 1974). However, the ratio of their specific activities changes with purification and the activities of this enzyme preparation respond differently to 1 mM ammonium sulfate. Ascorbate sulfatase activity for partly purified enzyme is activated 5-fold, while arylsulfatase activity is inhibited (Carlson, 1974). Ascorbate sulfatase purified 40 000-fold has been shown to be powerfully inhibited by phosphate and ascorbate 2-phosphate (Carlson et al., 1976). These results raised some questions: Is there a specific ascorbate sulfatase which can be separated from arylsulfatase? What are its properties? To answer these questions bovine ascorbate sulfatase was purified to homogeneity using procedures which are specific for isolating an ascorbate 2-sulfate hydrolyzing enzyme rather than procedures based on purifying arylsulfatase A. Physical and kinetic characteristics of this enzyme were determined.

Materials and Methods

Materials. Dipotassium L-ascorbate 2-sulfate was prepared in our laboratory by previously described methods (Tolbert et al., 1975). Trisodium L-ascorbate 2-phosphate was prepared by methods generously supplied by Dr. Paul Seib of Kansas State University, Manhattan, Kansas. Nucleotides were purchased from Calbiochem and Sigma Chemical Co. D-Glucose 6-phosphate, D-fructose 6-phosphate, glucose 6-sul-

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fate, and chondroitin sulfate were purchased from Sigma Chemical Co.

Ascorbate Sulfatase Assay. The assay consists of following the reduction of 2,6-dichloroindophenol (DCIP)[1] by the ascorbic acid produced from the enzymic hydrolysis of ascorbate 2-sulfate. On reduction DCIP changes from a blue to a colorless molecule. The decrease in absorbance is followed at 516 nm, the isosbestic point. The molar extinction coefficient of the potassium salt of DCIP at 516 nm is 9300. Two and one-half milliliters of a 0.15 mM DCIP solution in 0.1 M acetate buffer (pH 4.8) was added to a 4-mL cuvette. To the cuvette was added 0.5 mL of 40 mM dipotassium ascorbate 2-sulfate. The decrease in absorbance with time was measured before adding the enzyme since there is a slight acid-catalyzed ascorbate 2-sulfate hydrolysis at pH 4.8. Up to 0.2 mL of the enzyme fraction was added and the decrease in absorbance at 516 nm was followed using a recording spectrophotometer. No decrease in absorbance was detected in the presence of enzyme without ascorbate 2-sulfate. Enzyme fractions boiled prior to assay show no measurable activity.

The ascorbate sulfatase assay procedure in crude homogenates requires some modification since there is a low concentration of enzyme, a great deal of protein precipitation and nonspecific reduction of the DCIP. Two and one-half milliliters of the same DCIP solution were added to 0.05 or 0.10 mL of crude dialyzed homogenate. This solution was allowed to stand for 10 to 15 min and was then centrifuged. The supernatant was poured into a 3-mL cuvette. Substrate, 0.5 mL, was added and the decrease in absorbance at 516 nm was followed. The control reactions without the addition of substrate and the nonenzyme hydrolysis of ascorbate 2-sulfate were subtracted from the experimental assay. During purification all assays were done using enzyme samples which had been dialyzed against cold 5 mM Tris base, pH 7.0.

For kinetic studies it was necessary to maintain a constant ionic strength and therefore the assay was modified as follows. One and one-half milliliter of 0.15 mM 2,6-dichloroindophenol in 0.05 M KAc (pH 4.8) was placed in a 3-mL cuvette. To this was added an appropriate amount of 100 mM dipotassium ascorbate 2-sulfate, 0.5 M KAc (pH 4.8), and water to give a total volume of 2 mL and a final ionic strength of 0.1. The rate of reaction at each substrate concentration was measured several times. Velocities were calculated during the first 6 to 10 min of hydrolysis. The rate of ascorbate 2-sulfate hydrolysis is calculated from a line drawn tangent to the initial progress curve. In most cases the hydrolysis rate did not change appreciably during the first 6 to 10 min. The assay is continuous and linear with enzyme concentration up to reaction rates giving 7.0 nmol ascorbic acid/min. One unit of activity is defined as that amount of enzyme which will hydrolyze 1 μmol of ascorbate 2-sulfate per min. Typical changes in absorbance would be from a change of 0.02 absorbance unit/5 min to about 0.2 absorbance unit/5 min.

Arylsulfatase Assay. The procedure is similar to a previously described procedure (Nichol and Roy, 1964). Up to 0.2 mL of the enzyme fraction was added to 5 mL of a solution of 3.0 mM *p*-nitrocatechol sulfate in 0.1 M acetate buffer at pH 4.9. This solution was incubated at 37 °C for 1 h. One-half milliliter of this solution was placed in 5.0 mL of 0.2 N NaOH and the absorbance at 510 nm was measured. The molar extinction coefficient of the nitrocatechol anion is 12 500. A control was run without enzyme, and no detectable amount of the nitrocatechol anion was observed. This assay is point by point and is not linear with enzyme concentration. The enzyme concentration of each assay was adjusted so that the 510-nm absorbance was maintained at about 0.12.

For kinetic studies it was necessary to modify the assay procedure in order to maintain a constant ionic strength and to obtain reaction rates during the first 10 min of hydrolysis. Solutions of 50 mM dipotassium nitrocatechol sulfate and 0.5 M acetate buffer made pH 4.8 were prepared. Using these solutions and distilled water, the desired nitrocatechol sulfate concentrations were prepared by dilution in test tubes and maintained at a final ionic strength of 0.1. The total volume at each substrate concentration was 1 mL. Fifty microliters of the enzyme preparation was added and the reaction mixture was incubated for 10 min at 37 °C. Three milliliters of 0.2 N NaOH was then added and the absorbance at 510 nm was measured. Several determinations were made at each substrate concentration. When determining the pH optimum, the pH was adjusted using acetic acid or KOH. The substrate concentration during the pH optimum determination was 3 mM. Nitrocatechol sulfate hydrolysis progress curves show that the rate of hydrolysis decreases significantly during the 10-min incubation time. Thus a K_m for nitrocatechol sulfate was also determined by using initial velocities calculated from progress curves according to the method of Stinshoff (1972). The ionic strength, pH, and temperature of the assay were as described above.

Calculation of the K_m and K_i Values. K_m and K_i values were calculated from the 1/*s* intercept of Lineweaver–Burk plots. Each K_m and K_i value was computed from a linear regression fit of the data points. For each K_m value, three to four determinations were made and the average value and standard deviation from the average were computed. For K_i values, three to four determinations were made at each of two inhibitor concentrations and the average value and standard deviation from the average computed.

Protein Determination. Protein concentrations were determined by the method of Schaeterle and Pollack using bovine serum albumin as the standard (Schaeterle and Pollack, 1973).

Ascorbate Sulfatase Purification Procedure. Twenty kilograms of fresh bovine liver was obtained, stored frozen, and processed within 2 weeks. All steps in the purification procedure are done at about 5 °C.

Step 1: pH Fractionation. One kilogram of liver was homogenized in 1800 mL of cold distilled water. The homogenate was adjusted to pH 5.9 using 2 M acetic acid and centrifuged at 13 000*g* for 45 min. The supernatant was adjusted to pH 4.7 using 2 M acetic acid and centrifuged at 13 000*g* for 15 to 20 min. The sediment was suspended in 500 mL of cold distilled water and adjusted to pH 7.0 with 0.2 N NaOH. Four kilograms of liver was processed and the combined solutions were stirred for 30 min after the last solution was added. Time for processing 4 kg of liver to this point was about 2 h.

Step 2: Ammonium Sulfate Fractionation. The above solution was made 20% ammonium sulfate (114 g/L), centrifuged at 13 000*g* for 30 min and the precipitate was discarded. The ammonium sulfate concentration of the supernatant was increased to 55% (225 g/L) and allowed to stand overnight. The solution was centrifuged at 13 000*g* for 20 min and the supernatant was discarded. The sediment was collected and stored in the freezer. Twenty kilograms of liver was processed through this step before going on. The enzyme is stable indefinitely in ammonium sulfate solution, or suspension.

Step 3: Hydroxylapatite Column Chromatography. The precipitate from 20 kg of liver was dissolved in 1 L of distilled

[1] Abbreviations used: DCIP, 2,6-dichloroindophenol; Tris, trishydroxymethylaminomethane; AcO, acetate; EDTA, ethylenediaminetetraacetic acid.

water and dialyzed overnight against 55 L of 5 mM Tris base adjusted to pH 7.0 with HCl. After dialysis the solution was centrifuged at 20 000*g* for 1 h to remove any sediment. Two hundred grams of Bio-Rad HTP hydroxylapatite was prepared according to Bio-Rad instructions in 0.1 M acetate buffer (pH 5.0). A 3.5 × 57 cm column was prepared and washed with about 1.5 L of 0.1 M acetate buffer (pH 5.0). One-third of the enzyme preparation was applied to this column. The column was washed with 0.1 M acetate buffer (pH 5.0) until the effluent showed negligible absorbance at 280 nm. This effluent had no measurable ascorbate sulfatase activity. The enzyme activity was eluted from the column with 0.3 M ammonium sulfate in 0.1 M acetate buffer, pH 5.0. The column was stripped of protein with 0.4 M phosphate adjusted to pH 5.4 with acetic acid. Elution fractions, 50 mL each, were dialyzed against cold 5 mM Tris (pH 7.0) and assayed. Protein concentration was monitored by following the 280-nm absorbance. After each column run, the hydroxylapatite was removed from the column, washed with several volumes of 0.4 M phosphate, and then the column was repacked.

Step 4: Sephadex A-50 Column Chromatography. The ascorbate sulfatase activity from the hydroxylapatite columns was combined and concentrated by ultrafiltration from a volume of 1.8 L to 108 mL. The concentrated solution was made 0.1 M acetate (pH 5.0) using 1.0 M acetate buffer (pH 5.0). The enzyme solution was applied to a 2.5 × 55 cm Sephadex A-50 column which had been equilibrated with 0.1 M acetate buffer (pH 5.0). The enzyme was eluted with 1 L of a NaCl gradient, 0 to 0.4 M NaCl, prepared in 0.1 M acetate buffer (pH 5.0).

Step 5: A Second Hydroxylapatite Column. A small hydroxylapatite column with a bed volume of 10 mL was prepared as described above. The enzyme solution was dialyzed against 25 L of cold 5 mM Tris (pH 7.0) and applied to the column. The column was washed with 50 mL of 0.1 M acetate buffer (pH 5.0). The enzyme was eluted with 40 mL of 0 to 0.4 M ammonium sulfate gradient in 0.1 M acetate buffer (pH 5.0). Tenfold dilutions of each fraction were assayed for enzyme activity. Figure 1 shows the elution profile of a typical hydroxylapatite column. The ascorbate sulfatase activity was pooled and dialyzed against cold 5 mM Tris (pH 7.0). Gel electrophoresis of the protein at this point showed that there was a heavy protein species present in addition to the enzyme band.

Step 6: Preparative Ultracentrifugation. The enzyme solution was concentrated to 3 mL by ultrafiltration and layered on two 5–20% sucrose gradients prepared in 0.375 M Tris buffer (pH 8.8). The tubes were centrifuged at 24 000*g* for 40 h and punctured, and 1-mL fractions were collected. Gel electrophoresis of the fractions showed that the heavy protein separated from the enzyme.

Some preliminary data reported here were obtained using enzyme which had been partially purified using the procedure of Nichol and Roy through the acetone cut (Nichol and Roy, 1964), or by ammonium sulfate reverse phase solubility chromatography (Carlson, 1974). Ammonium sulfate reverse-phase solubility chromatography has been used in the purification of arylsulfatase A from human urine (Stevens et al., 1975).

Gel Electrophoresis Procedures: General Procedures. Gel electrophoresis was carried out using a Tris–glycine system (Ornstein, 1964; Laemmli, 1970) and an Ortec 4100 pulsed power supply. All the gels were slab gels 1 mm thick × 20 cm long.

Staining Procedures. The gels were stained for protein using Coomassie Brilliant Blue R, purchased from Sigma Chemical Co. (Fairbanks et al., 1971). The gels were stained for glycoprotein using Alcian Blue, purchased from Kodak (Wardi and Michos, 1972). Gels were stained for arylsulfatase activity by placing the gel in a solution of 3 mM nitrocatechol sulfate, which was 0.1 M acetate (pH 4.9), for 30 min at 37 °C. The gel was removed from the nitrocatechol sulfate solution and placed in 0.2 N NaOH. A bright red band, due to the nitrocatechol anion, appears where the enzyme is located and quickly diffuses. Gels were stained for ascorbate sulfatase activity by placing them in a 10 mM solution of dipotassium ascorbate 2-sulfate, which was 0.1 M acetate (pH 4.8), for 30 to 45 min at room temperature. Then the gel was placed in 0.5 M $AgNO_3$ in the dark. A metallic silver band appears at the location of the enzyme and does not diffuse. The gel can be placed in distilled water to remove excess $AgNO_3$.

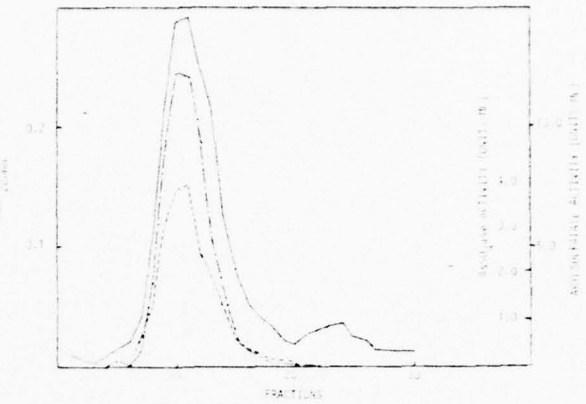

FIGURE 1: The elution pattern of the second hydroxylapatite column. Each fraction has a volume of 3 mL. (—) The 280-nm absorbance; (- - - -) ascorbate sulfatase; (- · - · -) arylsulfatase.

Results

The results of the purification procedure are summarized in Table I. The liver homogenate has a large amount of arylsulfatase activity relative to the ascorbate sulfatase activity. This is reflected in the low ratio of ascorbate sulfatase to arylsulfatase specific activities. After the pH fractionation, there is a large increase in this ratio. These data suggest that pH fractionation enriches sulfatase enzymes which hydrolyze ascorbate 2-sulfate. The supernatant from the pH 4.7 precipitation step contains arylsulfatase activity but has no measurable ascorbate sulfatase activity. Homogeneous bovine ascorbate sulfatase has arylsulfatase activity. Table I also shows an increase in yield of both activities after ammonium sulfate fractionation. The reason for the effect is not known, but was observed repeatedly. The 70 000-fold purification necessary to obtain homogeneous ascorbate sulfatase shows that levels of this enzyme in liver are quite low.

Gel electrophoresis of ascorbate sulfatase shows that both ascorbate sulfatase and arylsulfatase activities have identical protein and activity banding patterns. This pattern consists of a major sharp band located between the bovine serum albumin dimer and monomer bands followed by a light streaking pattern. This streaking pattern was seen repeatedly. It was noted that, when the enzyme preparation was exposed to pH 8.3 or pH 8.8 conditions for 2 to 3 days, this banding pattern shifted so that the streaking portion of the pattern became more dominant. This streaking pattern can be seen in the first dimension of the two-dimensional gel shown in Figure 2. This suggested that perhaps there was pH-dependent aggregation of the enzyme. Subsequent gels have shown that the lower

TABLE II: Ascorbate Sulfatase Purification.

	Units (μmol/min)			Spec. Act.		Purification		
	Aryl-sulfatase	Ascorbate sulfatase	Protein (mg)	Aryl-sulfatase	Ascorbate sulfatase	Aryl-sulfatase	Ascorbate sulfatase	Ratio[a]
Crude liver homogenate	4400[b]	525[b]	2.3×10^{6}[b]	0.0019	0.00023			0.12
Step 1: pH fractionation	690	480	2.7×10^{5}	0.0026	0.0018	1.4	7.8	0.69
Step 2: ammonium sulfate fractionation	1330	745	4.0×10^{4}	0.034	0.019	18	83	0.56
Step 3: hydroxylapatite column chromatography	306	400	100	3.1	4.0	1630	17 400	1.3
Step 4: Sephadex A-50 column chromatography	230	390	55	4.2	7.1	2200	31 000	1.7
Step 5: hydroxylapatite column chromatography	150	150	15	7.1–10.0[c]	8.2–10.0[c]	3700–5300[c]	36 000–45 000[c]	1.0
Step 6: preparative ultracentrifugation	31	40	3	11–13[c]	13–16[c]	5800–6800[c]	57 000–70 000[c]	1.2

[a] The ratio is the ascorbate sulfatase to arylsulfatase specific activity ratio. [b] These values are average values determined from several 1-kg crude liver homogenates. The values are then extrapolated to the values for 20 kg of liver. [c] The two values are representative of two 20-kg liver preparations.

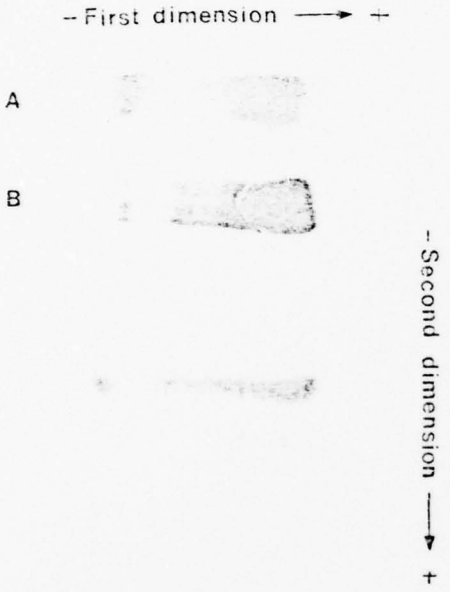

FIGURE 2: Two-dimensional gel electrophoresis of homogeneous bovine ascorbate sulfatase. The conditions for the first dimension are as follows. The stacking gel was 5% acrylamide in 0.375 M Tris buffer which is pH 8.8. The running gel was 10% acrylamide at pH 8.8. One hundred volts at 100 PPS was applied to the gel for 18.5 h. The initial current was 20 mA and the final current was 15 mA. Section A was stained for ascorbate sulfatase activity. Section B was stained for protein. A third section was electrophoresed in the second dimension. Each section contains 50 μg of ascorbate sulfatase. The second dimension was sodium dodecyl sulfate/8 M urea gel in Tris–glycine. The third section of the first dimension was placed in a solution of 0.125 M Tris (pH 6.8), 2% β-mercaptoethanol, 8 M urea, and 2% sodium dodecyl sulfate at 37 °C for 2 h. This section was placed on top of a running gel of 10% acrylamide prepared in 0.1% sodium dodecyl sulfate, 8 M urea, and 0.325 M Tris-HCl (pH 8.8). A 10% acrylamide capping gel prepared in 0.1% sodium dodecyl sulfate, 8 M urea, and 0.125 M Tris-HCl (pH 6.8) was poured on top of the 10% running gel. One hundred volts at 100 PPS was applied to the gel for 18 h. The initial current was 20 mA and the final current was 10 mA. The gel was stained for protein.

streaking band becomes the dominant band when the pH of the stacking gel is 8.8 and that the upper band is dominant when the stacking gel is pH 6.8. Only the upper band is present when gel electrophoresis is done in which the gel is pH 7.2 throughout. These gels are prepared and electrophoresed in 0.15 M Tris (pH 7.2). These results indicate that there is a shift in the aggregation state of the enzyme which is dependent upon pH. A sizing column, using Bio-Gel P-300 in 25 mM Tris (pH 8.0) and impure enzyme, gives a molecular weight for ascorbate sulfatase of 138 000. Another sizing column, using Agarose A-1.5m in 0.1 M acetate (pH 5.4) buffer and impure enzyme, gives a molecular weight of about 300 000 for ascorbate sulfatase. Both sizing column and gel electrophoresis experiments indicate that ascorbate sulfatase changes aggregation states depending upon pH. The kinetics responsible for the shift in aggregation state was not investigated further.

Gels of ascorbate sulfatase, run in the Tris–glycine system in the presence of 0.1% sodium dodecyl sulfate and 8 M urea, show the presence of two protein bands. These two protein bands have relative mobilities corresponding to molecular weights of 59 000 and 52 000. The 59 000 molecular weight band is more highly stained than the 52 000 molecular weight band. Both bands stain positively for glycoproteins. To determine whether one of these two protein bands could be an impurity, a two-dimensional gel was run. The first dimension was run under nondenaturing conditions where the pH of the stacking gel and running gel was 8.8. The second dimension was run under denaturing conditions. This gel, pictured in Figure 2, shows that the two bands seen under denaturing conditions are everywhere associated with the enzyme activity and the protein banding patterns observed in the first dimension. These results indicate that the ascorbate sulfatase activity is homogeneous even though it consists of two closely associated protein species.

The pH optimum of homogeneous ascorbate sulfatase is 4.8 at ionic strengths of 0.1 and 0.2. The pH optimum of ascorbate sulfatase partially purified through the acetone cut using the procedure of Nichol and Roy is 5.4 (Nichol and Roy, 1964). The pH optimum of the arylsulfatase activity of homogeneous bovine ascorbate sulfatase is pH 5.6 to pH 5.7.

Table II shows the effect of several compounds on ascorbate sulfatase activity. There appears to be a slight activation by $Mg(AcO)_2$, $Mn(AcO)_2$, and $ZnCl_2$. There is a slight inhibition by NaCl. Ascorbate 2-phosphate is the most powerful inhibitor followed by Na_2HPO_4 and then by the nucleotide triphosphates. It is possible that the nucleotide triphosphate inhibition is due, in part, to the presence of some inorganic phosphate. However, ascorbate 2-phosphate contains less than 0.1% inorganic phosphate. Glucose 6-sulfate and chondroitin sulfate are very poor inhibitors which suggests that they probably are poor substrates. EDTA does not affect ascorbate sulfatase indicating that divalent metal ions are not necessary for en-

TABLE II: The Effect of Various Compounds on Ascorbate Sulfatase Activity.

Reagent (1 mM)	% inhibition (% activation)
Na_2HPO_4	99
GTP	98
CTP	94
ATP	88
ADP	68
UMP	18
CMP	6
D-Glucose-6-PO_4	65
D-Fructose-6-PO_4	92
Ascorbate-2-PO_4	100
Na_2SO_3	77
Na_2AsO_3	63
Glucose-6-SO_4	0
Chondroitin-SO_4 (1 mg/mL)	6
Galactose	0
NaCl	4
LiCl	0
NH_4AcO	0
$Mg(AcO)_2$	(8)
$Mn(AcO)_2$	(13)
$ZnCl_2$	(6)
EDTA	0

zyme activity. Similar effects have been reported for the inhibition by sugar phosphates and by nucleotides of ascorbate sulfatase partially purified from the marine gastropod *Charonia lampas* (Hatanaka et al., 1975b). Bovine ascorbate sulfatase does not hydrolyze ascorbate 2-phosphate; however, *E. coli* alkaline phosphatase does hydrolyze ascorbate 2-phosphate (unpublished data). Phosphate inhibition of other sulfatase activities has not been studied in any detail.

The kinetics of ascorbate sulfatase depend upon ionic strength. At an ionic strength of 0.2 there is apparent substrate inhibition beginning at 4 to 5 mM ascorbate 2-sulfate. At ionic strength 0.1 substrate inhibition is not observed. At an ionic strength of 0.1 the arylsulfatase activity of pure ascorbate sulfatase is also substrate inhibited. This can be seen in Figure 3 where the Lineweaver–Burk plot results in an upward curving line near the $1/v$ intercept.

The kinetic properties of ascorbate sulfatase are summarized in Table III. The third and fourth columns of Table III compare the hydrolysis of ascorbate 2-sulfate with isoascorbate 2-sulfate. The enzyme preparation and the assay conditions are given in Table III. Ascorbate 2-phosphate competitively inhibits the hydrolysis of isoascorbate 2-sulfate and ascorbate 2-sulfate with a K_i of about 0.4 μM. The type of inhibition by inorganic phosphate is not clearly competitive or noncompetitive for either substrate using this enzyme preparation; Na_2HPO_4 inhibition of the pure enzyme is competitive with a K_i of 3.3 μM as recorded in the first column of Table III. The K_m for isoascorbate 2-sulfate hydrolysis is about 13-fold lower than the K_m for ascorbate 2-sulfate. Not given in Table III are data which show that the V_{max} for isoascorbate 2-sulfate hydrolysis is 16-fold lower than the V_{max} for ascorbate 2-sulfate hydrolysis. The K_m for ascorbate 2-sulfate after freezing and thawing this enzyme preparation is 2.9 mM. Ascorbate 2-sulfate hydrolysis is competitively inhibited by inorganic sulfate with a K_i of 0.04 mM.

The first and second columns of Table III compare ascorbate 2-sulfate hydrolysis with nitrocatechol sulfate hydrolysis. These studies were done using the homogeneous enzyme. The assay conditions are given above and stated briefly in Table III. Inhibition is competitive for all cases except for phosphate inhibition of the arylsulfatase activity. The Lineweaver–Burk plot for phosphate inhibition of the arylsulfatase activity resulted in upward curving lines, as shown in Figure 3. K_m and K_i values given in Table III are significantly higher for arylsulfatase activity than for the ascorbate sulfatase activity. The K_m for nitrocatechol sulfate calculated from initial velocities (Stinshoff, 1972) is 0.32 mM ± 0.02 mM which is not significantly different from the K_m of 0.5 mM ± 0.3 mM (see Table III) which was calculated from 10-min velocities.

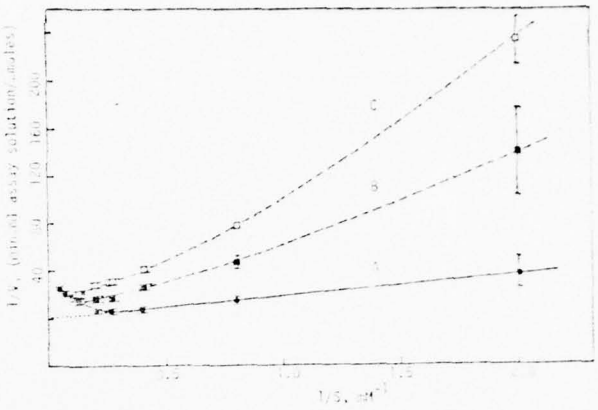

FIGURE 3: A Lineweaver–Burk plot of the arylsulfatase activity of ascorbate sulfatase and its inhibition by phosphate. The protein concentration of each assay is 0.37 μg/mL. The temperature is 37 °C and the ionic strength is 0.1. (A) Without phosphate; (B) with 25 μM Na_2HPO_4; and (C) with 50 μM Na_2HPO_4.

Discussion

The major purification step of ascorbate sulfatase is hydroxylapatite column chromatography. It was discovered earlier that ascorbate sulfatase is powerfully inhibited by inorganic phosphate (Carlson et al., 1976). Several methods of purification were attempted based on this inhibition. Hydroxylapatite was chosen since it is an inorganic phosphate material. The enzyme bound to the hydroxylapatite and could be removed with low phosphate concentrations (10 mM) or with 0.3 M ammonium sulfate. Under these conditions very little of the protein is removed from the column but most of the enzyme activity is removed. Ammonium sulfate was chosen to elute the enzyme because it is a less powerful inhibitor than inorganic phosphate and therefore facilitates the assaying of the fractions. The second hydroxylapatite column (step 5) results in very little purification, gives a poor yield, and is probably an unnecessary step.

pH fractionation enriches sulfatase enzymes which hydrolyze both ascorbate 2-sulfate and nitrocatechol sulfate. The literature supports the idea that pH fractionation may remove arylsulfatase activity which does not hydrolyze ascorbate 2-sulfate. Ascorbate 2-sulfate is a very poor substrate for arylsulfatase *B*, while arylsulfatase *A* readily hydrolyzes this molecule (Roy, 1975). At pH 4.7 bovine arylsulfatase A precipitates while arylsulfatase B remains in solution (Nichol and Roy, 1964; Allen and Roy, 1968). Thus it is possible that sulfatase enzymes which hydrolyze ascorbate 2-sulfate are separated from sulfatase enzymes which do not hydrolyze ascorbate 2-sulfate during the pH fractionation.

A purification procedure based on concanavalin A-Sepharose affinity chromatography was attempted (unpublished data). Arylsulfatase from sheep brain has been purified using this method (Bishayee et al., 1973; Bishayee and Bachhawat, 1974). Ascorbate sulfatase binds to concanavalin A-Sepharose and could not be removed using a variety of eluents and con-

TABLE III: Kinetic Properties of Ascorbate-2-sulfate Sulfohydrolase.

Substrate or inhibitor	Ascorbate sulfatase act.,[a] ascorbate 2-sulfate hydrolysis	Arylsulfatase act.,[a] nitrocatechol sulfate hydrolysis	Ascorbate 2-sulfate[b] hydrolysis	Isoascorbate 2-sulfate[b] hydrolysis
NCS	K_i = 0.09 ± 0.01 mM	K_m = 0.50 ± 0.3 mM		
As-2-SO_4	K_m = 1.2 ± 0.2 mM[c]	K_i = 3.8 ± 1.8 mM	K_m = 1.3 ± 3 mM[d]	
As-2-PO_4	K_i = 0.5 ± 0.2 μM	K_i = 1.8 ± 0.5 μM	K_i = 0.4 ± 0.4 μM	K_i = 0.4 ± 0.1 μM
Na_2HPO_4	K_i = 3.3 ± 0.9 μM	K_i = [e]	[Na_2HPO_4] at 50% inhibition is 8 μM[f]	[Na_2HPO_4] at 50% inhibition is 8 μM[f]
Iso-As-2-SO_4				K_m = 1.1 ± 0.1 mM
Na_2SO_4			K_i = 0.04 ± 0.01 mM	

[a] These studies were done using pure enzyme. The conditions were: temperature 37 °C, ionic strength 0.1; pH 4.8. [b] These studies were done using enzyme purified through the second hydroxylapatite column. The conditions were: temperature 25 °C, ionic strength 0.1, and pH 4.8. [c] The K_m of the pure enzyme at a temperature of 25 °C, ionic strength 0.1, and pH 4.8 is 0.5 ± 0.1 mM. [d] The K_m of the enzyme at this stage of purification is 2.9 ± 1.1 mM after freezing and thawing. [e] The Lineweaver–Burk plot of phosphate inhibition of the arylsulfatase activity results in upward curving lines, and the K_i cannot be determined by this type of plot. [f] Inhibition is 50% at ascorbate 2-sulfate concentration of 4 to 6 mM. Inhibition is 50% at an isoascorbate 2-sulfate concentration of 0.9 mM.

ditions. It is not known whether the column destroyed the enzyme activity; however, concanavalin A, in concentrations up to 0.6 mg/mL, does not inhibit ascorbate sulfatase activity. The binding of the enzyme suggests that it is a glycoprotein.

Initially, it was hoped that there would be an ascorbate sulfatase enzyme which would prove to be distinct from arylsulfatase, and which could be separated from arylsulfatase activity during purification. However, arylsulfatase and ascorbate sulfatase activities copurify to homogeneity. Several enzymic properties of ascorbate sulfatase change during purification. These include a change from activation to inhibition of the enzyme by sulfate, changes in K_m values, and changes in the pH optimum. Therefore there is good reason to believe that the in vivo enzyme is different from the homogeneous enzyme. Arylsulfatase A, prepared by Nichol and Roy (1964), is probably also a modified enzyme and differences between ascorbate sulfatase and arylsulfatase A could rise from the fact that the homogeneous preparations are different. Whether ascorbate sulfatase and arylsulfatase A are the same enzyme or have a common origin is unknown. Similarities between the two enzymes are discussed below. The data do not rule out the possibility that there is an ascorbate sulfatase enzyme which does not have arylsulfatase activity. Such an enzyme would be difficult to purify because it would be masked by the enzyme purified here which has both ascorbate sulfatase and arylsulfatase activities.

Gel electrophoresis and sizing columns show that ascorbate sulfatase changes aggregation states depending upon pH. These gels also show that both ascorbate sulfatase and arylsulfatase activities are associated with the same protein species. pH dependent changes in aggregation states have been described for bovine arylsulfatase A. Ultracentrifugation studies show that arylsulfatase A changes from a molecular weight of 100 000–120 000 at pH 7.5 to a molecular weight of about 400 000 at pH 5.5 (Nichol and Roy, 1964, 1965).

Gels run under denaturing conditions, i.e., sodium dodecyl sulfate and 8 M urea, and two-dimensional gel electrophoresis shows that ascorbate sulfatase is pure and that it consists of two distinct protein species. These protein species consist of a dark staining Coomassie blue band with a molecular weight of 59 000 and a light staining Coomassie blue band with a molecular weight of 52 000. The difference in the intensity of staining is not understood. If the intensity of staining is proportional to the amount of each protein species, then the two proteins are not present in a reasonable ratio for the enzyme to be a simple two subunit enzyme. These data suggest that bovine ascorbate sulfatase is a complex enzyme physically. Ascorbate sulfatase has similar physical properties to arylsulfatase A.

Differences between the kinetic characteristics of nitrocatechol sulfate hydrolysis with those of ascorbate 2-sulfate hydrolysis shown in Table III suggest that the enzymic mechanism of ascorbate sulfatase is more complex than a simple one-site model mechanism. A simple one-site model would dictate that the K_m for ascorbate 2-sulfate equal the K_i value for ascorbate 2-sulfate inhibition of the arylsulfatase activity. They are significantly different. The K_i values for ascorbate 2-phosphate inhibition of both arylsulfatase and ascorbate sulfatase activities should be equal, but are not. The K_m for nitrocatechol sulfate should equal the K_i for nitrocatechol sulfate inhibition of the ascorbate sulfatase activity. Again, they are not equal. One explanation of these data could be that the enzyme has multiple unequivalent binding sites for both nitrocatechol sulfate and ascorbate 2-sulfate. Other data presented in this paper also suggest that the enzyme has multiple binding sites. The nonlinear Lineweaver–Burk plot for phosphate inhibition of the arylsulfatase activity is suggestive of multiple binding sites. Substrate inhibition by both ascorbate 2-sulfate and nitrocatechol sulfate suggests multiple binding sites for these molecules. Thus, ascorbate sulfatase is a complex enzyme kinetically as well as physically. Further work is necessary to understand the kinetic and physical complexity of this enzyme. Attention should be focused on the relationship of the two protein species which comprise ascorbate sulfatase and to the fact that this enzyme is a glycoprotein.

It is known that ascorbate 2-sulfate is converted to ascorbic acid in several biological systems (Halver et al., 1975; Baker et al., 1975; Knight, 1974). The most obvious role for ascorbate sulfatase would be to carry out this conversion. However, from the data presented in this paper, it is not so obvious that ascorbate sulfatase in its present state would hydrolyze ascorbate 2-sulfate in vivo. The best estimates of urine and tissue homogenate levels of ascorbate 2-sulfate in the rat (March, 1972) are much lower than the K_m value. In addition, physiological concentrations of phosphate are much higher than the K_i value for phosphate of 3.3 μM. These data make it unlikely that purified ascorbate sulfatase could hydrolyze ascorbate 2-sulfate in vivo. It is possible, however, that microenvironmental concentrations of these molecules in vivo could be such that hydrolysis would take place. It is also possible that an ascorbate sulfatase exists in the trout which has quite different properties since in this species ascorbate 2-sulfate is an effective vitamin

substitute for ascorbic acid (Halver et al., 1975). Ascorbate sulfatase is a glycoprotein and as such it could be membrane associated where phosphate concentrations are low and ascorbate 2-sulfate hydrolysis could take place. The rate of ascorbate 2-sulfate hydrolysis in tissues is not known. Thus, the physiological significance of the data presented in this paper concerning the in vivo role of ascorbate sulfatase is uncertain.

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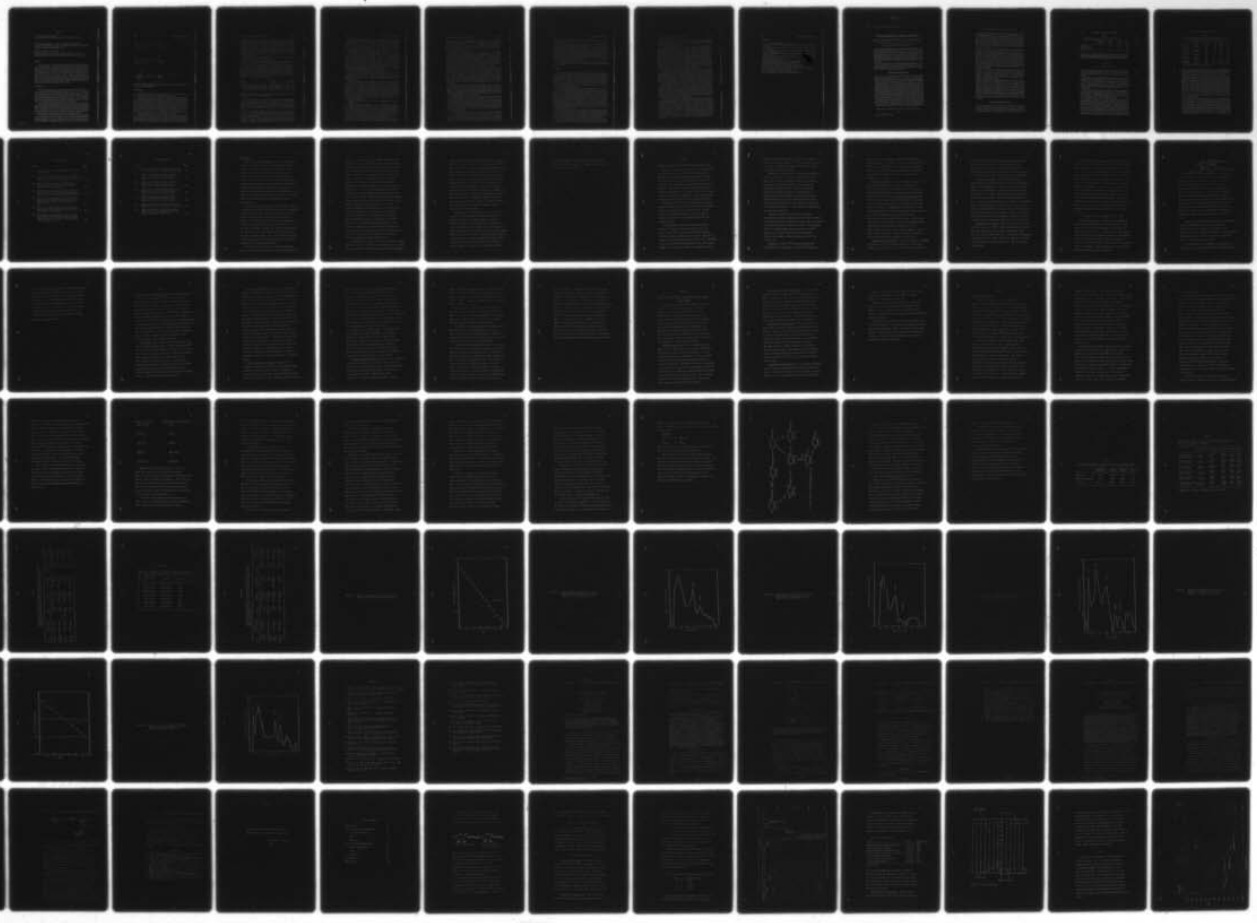

Part III

Carbohydrate Research, 60 (1978) 251–258

A NEW SYNTHESIS OF L-*threo*-HEX-2-ENARO-1,4-LACTONE ("SACCHAROASCORBIC" ACID); A METHOD FOR THE PROTECTION OF THE ENEDIOL OF ASCORBIC ACID

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ABSTRACT

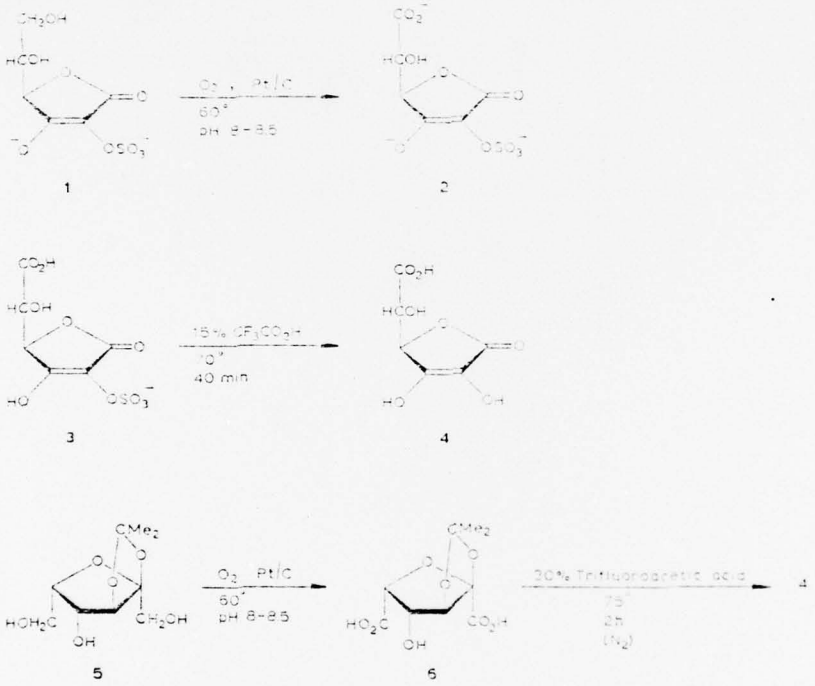
A new synthesis of L-*threo*-hex-2-enaro-1,4-lactone (**4**) ("saccharoascorbic acid") is presented, whose unique feature involves oxidation of the side chain of ascorbic acid. Ascorbate 2-sulfate (**1**) was selectively oxidized in water at pH 8–8.5 with platinum-on-carbon catalyst to yield the 2-sulfate (**3**) of **4**. Hydrolysis of **3** in 15% trifluoroacetic acid for 90 min at 70° yielded **4**. The procedure affords a useful preparation of **4**, and demonstrates the excellence of sulfation for protection of the enediol of ascorbic acid during synthetic manipulations of the side chain. The sulfated ring is stable to oxidizing agents and to base, yet sulfate is readily removed by acid hydrolysis. The properties of a new compound (**3**) of biological significance, and those of the previously uncharacterized **4**, are reported.

INTRODUCTION

The C-6 oxidized derivative of ascorbic acid, L-*threo*-hex-2-enaro-1,4-lactone[1] (**4**), was described in a 1947 patent by N. R. Trenner[2], who named the compound L-gulosaccharoascorbic acid. The patented synthesis involved oxidation of an *O*-isopropylidenesorbose derivative, with subsequent isomerization and lactonization. The product was identified only by its m.p. No further chemical or biological characterization of **4** has since been reported, although it is unique in being the only side-chain oxidized derivative of ascorbic acid to have been described.

Recent studies imply that C-6 oxidation of ascorbic acid is an important metabolic process[3] and compound **4** has been postulated as a metabolite of ascorbic acid[4], although **4** was not then available to verify this proposal. Hornig[5] has proposed that the major, polar metabolite of ascorbate 2-sulfate (**1**) observed in the urine of rats and guinea pigs is the 2-sulfate (**3**) of **4**. This compound has not been previously synthesized, nor has it been identified as a product from natural sources.

This paper presents a new synthesis of **4** in which **2** is prepared as an intermediate. Purification and characterization of both compounds is described. The synthesis introduces a procedure of potential general value for the protection and subsequent liberation of the labile ring-hydroxyl groups of ascorbic acid. We were



not able to obtain **4** by Trenner's patented procedure as written, and modifications of his synthesis are also outlined here.

EXPERIMENTAL

Preparation of potassium ascorbate 2-sulfate (K_2-**1**). — The salt K_2-**1** was prepared by a published procedure[6] developed in our laboratory.

Catalytic oxidation of **1**: *preparation of* **2**. — The potassium salt of **1** (K_2-**1**, (2.0 g, 6.0 mmol) was dissolved in 150 ml of doubly-distilled water and the pH adjusted to 8.5 with 0.5M potassium hydroxide. The solution was placed in a 250-ml, three-necked flask equipped with a paddle-type stirrer capable of 1700 r.p.m. (no load). A pH probe was placed in one neck of the flask and a buret filled with 0.5M potassium hydroxide was placed so that the base could be added dropwise during the reaction. The temperature was maintained at 60° by means of a water bath. Platinum-on-carbon catalyst (5%, 0.5 g, Matheson Coleman and Bell) was added to the solution and sweeping with oxygen (~0.5 l/min) was begun.

The pH decreased immediately, and was maintained between 8.0 and 8.5 by addition of 0.5M potassium hydroxide. The volume of alkali consumed in this manner was recorded. After 9 h, the reaction was stopped and the mixture was filtered through fine paper to remove the catalyst, which was recovered. The solution was

concentrated to 10 ml by rotary evaporation and centrifuged to remove the last traces of carbon. The pH was adjusted to 10 with potassium hydroxide, and the product was precipitated at 50° by addition of methanol. Filtration yielded 1.9 g of a non-hygroscopic powder. This crude product was dissolved in 10 ml of double-distilled water and warmed to 50°. Methanol was added dropwise until the stirred solution remained cloudy. Crystallization occurred on continued stirring, with cooling to room temperature. Filtration yielded colorless, non-hygroscopic crystals of the tripotassium salt of **2**: yield 1.75 g (70%), m.p. 240–245° dec., λ_{max} 254 nm (ε 16,800) at pH 6.86.

Anal. Calc. for $C_6H_3K_3O_{10}S \cdot 2H_2O$: (420.1) C, 17.13; H, 1.67; S, 7.64. Found (Huffman Laboratories): C, 17.21; H, 1.86; S, 7.31.

This oxidation was also performed as before but with 10.0 g of potassium **1** in 200 ml of water, to yield 7.5 g (60%) of tripotassium **2**, m.p. 240–245° dec.

Analytical methods for and properties of **2**. — Column chromatography on Whatman DE-32 (*O*-diethylaminoethyl) cellulose (sulfate form) was used as the primary assay for the purity of **2**. Elution was achieved by a pumped gradient of sulfuric acid, and the u.v. absorbance of the eluate was monitored in a flow cell. The recrystallized products showed no impurities registering >1% of the product peak on the u.v. monitor printout. The column separated **1** and **2**. Systems were developed for the separation of **1** and **2** by t.l.c.

Solvent	*T.l.c. media*	R_f**1**	R_f**2**
150:75:25 Propanol–ammonia–water (A)	Bakerflex F cellulose	0.60	0.45
150:75:25 Propanol–ammonia–water (A)	Eastman 6060 silica gel	0.59	0.47
150:75:50 Ethylacetate–acetic acid–water (B)	Eastman 6060 silica gel	0.22	0.17

Solvent *A* on cellulose plates was the most convenient of these systems, as the same solvent developed very slowly on silica gel plates.

The K_3-**2** and K_2-**1** salts were shown to be quite stable in aqueous solution at room temperature. Solutions of 10 mg of K_3-**2** and K_2-**1** in 4 ml of water were stored in stoppered flasks for 48 days. T.l.c. of the stored solutions showed no sign of decomposition. The solutions were not discolored, and the chromatograms showed no other spots or streaking, nor any sign of material at the origin or solvent front.

The CO_2H infrared absorption in K_3-**1** did not appear as a separate band. The group is present as a carboxylate anion, and its primary absorption caused by asymmetric stretching occurs at 1610 cm^{-1}, and thus is a part of the large C-2–C-3 double-bond stretching absorption shown by both compounds at 1620 cm^{-1}. The less-intense, symmetric, stretching absorption of the carboxylate anion does occur at 1400 cm^{-1}. Assignments for K_3-**2** and K_2-**1** are the same: 3520 (O-H), 3200–3400

broad (O-H), 1720 (ketone C=O), 1620 (C=C), and 1240 cm^{-1} (sulfate S-O stretching).

The salt K_3-**2** was quite soluble in water: 7.5 g dissolved in 10 ml of water at room temperature, but it was virtually insoluble in methanol, ethanol, chloroform, acetonitrile and acetone. Aqueous solutions of **2** gave a brick-red color in the presence of ferric ions indistinguishable from the color given by **1**. The intensity of the color makes this a useful spot test for the detection of the compounds. The salt K_3-**2** does not reduce 2,6-dichloroindophenol and gives a negative Tollens' test.

Acid-catalyzed hydrolysis of **2**: *preparation of* **4**. — The K_3 salt of **2** (2.40 g, 5.71 mmol) was dissolved in 10 ml of doubly-distilled water and passed through a 2.5 × 50 cm column of Dowex-50 resin (H^+ form, 20–40 mesh), with elution by distilled water. The fractions containing **2** were collected (volume = 40 ml). This solution was placed in a tube equipped with a nitrogen inlet, consisting of a Teflon tube inserted to the bottom of the solution, and the tube was then placed in a water bath at 75°. Sweeping with nitrogen was begun and 7 ml of trifluoroacetic acid was added. Sweeping with nitrogen was maintained throughout the reaction. Aliquots of 25 µl were removed at intervals and added to 10 ml of pH 6.86 buffer, and then titrated rapidly with standardized mM 2,6-dichloroindophenol.

A 1.0 mM solution of 2,6-dichloroindophenol was prepared from 0.145 g of its sodium salt in 500 ml of doubly-distilled water. Standardization was achieved by titration with three weighed samples of reagent-grade L-ascorbic acid. The endpoint in standardization and in the assay was judged to be the appearance of the first permanent blue color.

After 90 min the reaction was complete as determined by titration with 2,6-dichloroindophenol. The solution was chilled in ice and 1.12 g (5.71 mmol) of barium carbonate were added carefully (CO_2 evolved). After stirring for 10 min, the suspension was filtered through a medium-pore glass filter to remove precipitated barium sulfate. Evaporation to a syrup, addition of abs. ethanol (25 ml) and further evaporation to a syrup was followed by drying under vacuum. Crystallization from acetonitrile–chloroform gave a non-hygroscopic solid. Recrystallization from the same solvents yielded **4** as a white, microcrystalline solid: yield 0.70 g (64%), m.p. 212–215 dec., (lit.[2] 206–210°): λ_{max}^{KBr} 3530, 3300 broad, 1760, 1710, 1660 cm^{-1}; λ_{max} 266.5 nm (ε 10,200) at pH 6.86.

Anal. Calc. for $C_6H_6O_7$: C, 37.88; H, 3.15. Found (Huffman Laboratories): C, 38.24; H, 3.10.

Analytical methods for and properties of **4**. — Column chromatography on *O*-(diethylaminoethyl)cellulose (sulfate form) was used as the primary assay for the purity of **4**. Elution was achieved by pumped gradients of sulfuric acid, and the u.v. absorption of the eluant was monitored with a flow cell. The recrystallized products showed no impurities registering above 1% of the product peak area on the u.v. monitor printout. A small amount of an impurity in the crude product appeared as a peak at the void volume. The column separated **4** from ascorbic acid.

The identity of **4** as just obtained with that from Trenner's synthesis was demonstrated by chromatographic identity, their identical i.r. spectra, and an un-

changed mixed m.p. The carbonyl-stretching vibration in the i.r. spectrum of the carboxylic acid group in **4** appears at 1710 cm^{-1}, well distinguished from the 1760 cm^{-1} (lactone C=O) and 1660 cm^{-1} (C=C) absorptions seen for ascorbic and **4**. The acid **4** is quite soluble in water and shows appreciable solubility in acetonitrile. It is less soluble in methanol and ethanol, and is insoluble in chloroform. It reduces 2,6-dichloroindophenol solutions.

*Preparation of 2,3-O-isopropylidene-α-*L*-sorbofuranose* (**5**). — Finely ground L-sorbose (30 g, 0.17 mol) was added to 500 ml of reagent-grade acetone in a 1-liter round bottom flask. Concentrated sulfuric acid (30 ml) was added during 0.5 h to the stirred suspension. The temperature remained at 25 ± 3° and the sorbose did not completely dissolve in this time. After an additional h of stirring the solution was cooled in ice and neutralized with 50% sodium hydroxide, keeping the temperature below 20°. The precipitated sodium sulfate was filtered off and the solution concentrated (rotary evaporator) to remove acetone, the final volume being 80 ml.

The solution was poured into a solution of 40 g of concentrated sulfuric acid in 220 ml distilled water. This solution was stirred for 1 h at room temperature and then cooled with ice and neutralized with 50% sodium hydroxide at no warmer that 25°. Rotary evaporation to a syrup (volume ~25 ml) and extraction with four 30-ml portions of ethyl acetate was followed by concentration of the ethyl acetate extract to 60 ml. Compound **5** crystallized readily from this solution on standing, forming large white to transparent crystals, yield 7.6 g (20%); m.p. 92–93° (Fisher-Johns); λ_{max}^{KBr} 3450 broad (O-H), 2900 (C-H), 1390 and 1380 cm^{-1} (*gem*-dimethyl, C-H bending).

*Preparation of 2,3-O-isopropylidene-α-*L*-xylo-hexulosaric acid* (**6**). — Compound **5** (4 g, 18 mmol) was dissolved in 150 ml of double-distilled water. The pH was adjusted to 8.5 with potassium hydroxide and 0.5 g of platinum-on-carbon (Matheson Coleman and Bell) was added. During the reaction, the pH was maintained at 7.5 to 8.5 with 0.5M potassium hydroxide, and base consumed in this manner was recorded. The reaction temperature was maintained at 60°.

When consumption of base stopped, the hot suspension was filtered through fine filter-paper, yielding a colorless solution. No discoloration nor decrease in yields were observed in reactions conducted for up to 60 h. The solution was evaporated to 25 ml, chilled to 0°, and acidified to pH with cold, M hydrochloric acid. Extraction with four 30 ml portions of ethyl acetate and evaporation of the ethyl acetate fraction to 30 ml gave a solution from which the product **6** crystallized on being kept; yield 2.0 g (44%), m.p. 202–204° dec.; λ_{max}^{KBr} 3480, 1735 (C=O), 1380 and 1390 cm^{-1} (*gem*-dimethyl C-H bending).

Acid-catalyzed conversion of **6** *into* **4**. — A solution of 1.0 ml trifluoroacetic acid in 2.0 ml of double-distilled water in a test tube was placed in a water bath at 75°. Nitrogen was bubbled through the solution for 10 min prior to and during the course of the reaction by means of a Teflon tube extending to the bottom of the solution. Compound **6** (100 mg) was added, and it dissolved immediately. During

the reaction, 50 μl aliquots were removed and assayed for **4** by titration with 2,6-dichloroindophenol.

The 50-μl aliquots were added to 15 ml of pH 6.86 buffer and rapidly titrated with 2,6-dichloroindophenol. After 2.3 h, the reaction was complete, as determined by titration with 2,6-dichloroindophenol and the darkened solution was chilled with ice. Evaporation of the solution to ~0.5 ml, followed by addition of 1 ml of water and evaporation, gave a syrup that was vacuum-dried overnight to complete removal of trifluoroacetic acid. This material was dissolved in 2 ml of hot acetonitrile. Chilling precipitated **4** as a solid that was filtered off and dried: yield 21 mg (25%), m.p. 190–195° dec.; λ_{KBr}^{max} 3530, 3300, 1760, 1710, 1660 cm^{-1}. The solid from the dark acetonitrile solution was slightly tan in color.

Crude products thus obtained were recrystallized from acetonitrile to yield **4** as a white, microcrystalline solid, m.p. 210–214° dec.; average yield of recrystallized product was 28%.

DISCUSSION

The synthesis described in this paper gives L-*threo*-hex-2-enaro-1,4-lactone (**4**) in 45% yield from potassium ascorbate 2-sulfate (K_2-**1**). The preparation of K_2-**1** is a direct procedure, giving the product in 90% yield from ascorbic acid; this salt is also commercially available[7]. The synthesis of **4** requires only three steps from ascorbic acid, and it is not necessary to purify the K_3-**2** intermediate, as it is the sole product detected from the catalytic oxidation. The higher yield and ease of product isolation of the method are largely a result of the absence of an enediol-lactone, ring-closure step during the synthesis.

This procedure is the first reported synthetic oxidation of the side chain of ascorbic acid. The difficulty of performing any synthetic manipulation on the side chain of ascorbic acid arises from the presence of four reactive hydroxyl groups which must be distinguished chemically, and from the lability of the enediol to quite mild oxidizing agents. No method has previously been reported for the protection of the enediol during such a synthesis. The sulfation of ascorbic acid is an excellent method for the protection of the enediol ring as: (*a*) sulfate may be selectively introduced at the 2-position, (*b*) introduction proceeds in very high yield (90%), (*c*) compound **1** is stable to mild oxidation and to basic hydrolysis, and (*d*) sulfate is readily removed in excellent yield to give aqueous solutions of the modified ascorbate and readily precipitated sulfate ions.

The stability to atmosphere oxidation imparted to the enediol ring by protection as **1** is clearly demonstrated by this work. In contrast, ascorbic acid itself is rapidly oxidized, probably to the unstable dehydroascorbic acid, by the conditions of the catalytic oxidation. Compound **1** is stable to hydrolysis from pH 4–13, although at pH 4 it is slowly hydrolyzed and at pH 3 and below it is rapidly hydrolyzed.

There are limits to the stability of **1** to oxidizing agents. The oxidative desulfation of **1** in water by bromine was first observed by Ford and Ruoff[8]. The reaction

also occurs in *N,N*-dimethylformamide[9]. Treatment of the 5,6-isopropylidene acetal of **1** with *m*-chloroperoxybenzoic acid and 2,3-dichloro-5,6-dicyanobenzoquinone was reported to result in destitution[10].

It is possible that methods can be developed for the use of other protecting groups. The total insolubility of K_3-**1** in nonpolar and most polar solvents is a limitation that led us to seek an uncharged, nonpolar protecting group. Attempts were made to prepare the benzyl, methoxymethyl, and tetrahydropyranyl ethers of the ascorbate enols, but pure products were not isolated. The methoxymethyl and tetrahydropyranyl ethers should show acid-labile, base-stable properties, similar to that of ascorbate sulfate, whereas the benzyl ether might be stable to both mild acids and bases. Previously reported derivatives of ascorbic acid are not promising as protected forms of the molecule. The 3-methyl and 2,3-dimethyl ethers are hydrolyzed under rather severe conditions, and the 3-esters are rapidly hydrolyzed by neutral aqueous solutions[11]. 2-*O*-Acyl esters should be more stable, and might be useful as mild-acid stable, base-labile, protected forms of ascorbic acid.

The major modification made in repeating Trenner's synthesis was the use of 30% trifluoroacetic acid in the final isomerization in place of the concentrated hydrochloric acid reported in the patent. Attempts to carry out this conversion with concentrated hydrochloric acid resulted in decomposition of the intermediate. New conditions, using trifluoroacetic acid, were developed and optimized by monitoring the reaction through titrations with 2,6-dichloroindophenol. The product was obtained and recrystallized from acetonitrile instead of from 20% hydrochloric acid.

The catalytic oxidations were monitored by recording the volume of alkali required to maintain the *pH* at 8.5. One equivalent of acid is formed in the oxidation. The rates were initially very rapid, and then were followed by a slow approach to completion. Heyns[12] reported that such complex kinetics are normal for these oxidations. The reason for this behavior is not known, but a reversible inhibition, not poisoning, of the catalyst seems probable. Re-use of the catalysts in our reactions resulted again in rapid, initial rates, followed by a slow approach to completion. The catalysts were re-used in up to eight reactions.

Heyns and Paulsen have reviewed the selective oxidation of organic compounds[13] and specifically carbohydrates[12,14] up through 1962. The selectivity of the oxidation for the primary hydroxyl group of **1** is in accordance with other results in the literature. It was of interest to know whether the hydroxyl group at C-5 could be slowly oxidized to a ketone under the conditions of the catalytic oxidation. Extended exposure of K_3-**2** to the conditions of the catalytic oxidation resulted in formation of small proportions (5%) of a new, u.v.-absorbing product detected by column chromatography. The identity of the product is not known. We have found no report of the catalytic oxidation of an α-hydroxy acid to an α-keto acid, the most nearly analogous system being the conversion of an α-hydroxylactone to an α-ketolactone[15].

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Periodate Degradation of Labeled Ascorbic Acid[1]

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The oxidation of ascorbic acid has been studied by Juni and Heym (1), who showed that glyoxalate was formed slowly in 91% yield. More recently, Verma and Grover (2) have used periodate oxidation of ascorbic acid to formaldehyde followed by gravimetric assay of the dimedone–formaldehyde precipitate as the basis of an assay procedure for ascorbic acid. Horowitz and King (3) developed a degradation procedure for the osazone of labeled ascorbic acid which has been used in recent years by Loewus (4).

This paper is a report on a study of periodate oxidation of ascorbic acid using labeled ascorbic acid to establish definitively the C_1 and C_6 degradation products. This work is part of a larger study on the chemical nature of urinary metabolites of ascorbic acid.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Ascorbic acid was purchased from New England Nuclear Corporation. [6-^{14}C]Ascorbic was synthesized as described (5). Sodium [^{14}C]carbonate solution was prepared from standardized barium [^{14}C]carbonate. Potassium metaperiodate, 5,5-dimethyl-1,3-cyclohexandedione (dimedone), and ascorbic acid were reagent grade materials.

Periodate oxidation. A three necked 250 ml round bottom flask was charged with 180 ml of 0.11 M KIO_4, pH 8. The flask was fitted with a pH electrode, a 60 ml pressure compensated dropping funnel, a sweep gas inlet tube extending to the bottom of the flask, an exit tube leading to two 15 ml CO_2 absorption traps, and a stirring bar. The reaction vessel was covered with dark paper to exclude light, left at room temperature, and swept with N_2 at about two bubbles per second. The CO_2 traps were filled with ethanolamine. A 2.5 mM sample of L-ascorbic acid in 20 ml water was added from the funnel by drops over 15 min. The dropping funnel was washed with 2 × 2 ml of 2.5 M KOH. The reaction mixture was stirred for 1 hr. The pH ranged from 7.5 to 8.1. Five milliliters of concentrated HCl were added to the reaction mixture, and then 15 ml of 6

[1] Supported by a contract with The Surgeon General's Office of the U. S. Army.

M sodium arsenite was added by drops over 10 min. After the yellow color disappeared (about 10 min) the sweep was maintained for 1 hr.

Formaldehyde determination. The reaction mixture was diluted with water, and enough 5 M KOH was added to bring the final solution to pH 4.6 in 400 ml. A 20 ml aliquot was taken from the solution and 2 ml of dimedone solution (80 mg/ml 95% ethanol) was added. The solution stood overnight. The white precipitate was collected on a tared sintered glass filter, dried, and weighed. The melting point of the dimedone derivative was 191–192.5°.

Oxalate determination. The formaldehyde filtrate was neutralized with 5 M KOH, and 4 ml of 0.5 M calcium acetate was added. The solution stood for 1 hr. The calcium oxalate precipitate was collected on a fine sintered glass filter and washed with 5 ml water. The filtrate was removed for the formic acid determination. The precipitate was washed with 2 ml of 4 M acetic acid solution and this filtrate was discarded. The remaining precipitate was treated with 6 ml of 2 M HCl, filtered, and washed with 2 ml water. One milliliter of this solution was used for radioassay. The remaining solution was heated to 60° and the oxalic acid was determined by titration with 10^{-3} M standard potassium permanganate solution, using a pink end point.

Formic acid determination. The calcium oxalate filtrate was vacuum distilled to dryness at 34°. To the residue, 10 ml water was added, followed by 10% phosphoric acid added by drops until the solution reached a pH of 2. The solution was distilled to dryness *in vacuo*. Ten milliliters of water was added to the residue and the solution was distilled to dryness. The last step was repeated once.

The four distillates were combined and neutralized with 0.1 M KOH. The neutral solution was concentrated in vacuo to 3 ml and transferred with 15 ml wash water to a large test tube. Three milliliters of 7% mercuric chloride, 1 ml saturated sodium acetate, and 0.2 ml 4 M HCl were added. The tube was closed with a stopper containing a N_2 sweep inlet tube and an exit tube leading to two 15 ml CO_2 absorption bubblers filled with ethanolamine. The test tube was covered with black tape and heated in a boiling water bath for 1 hr with a N_2 sweep of about two bubbles per second. The test tube was cooled in an ice-water bath for 30 min. The calomel precipitate was collected, air dried on a tared glass filter, and weighed.

RESULTS AND DISCUSSION

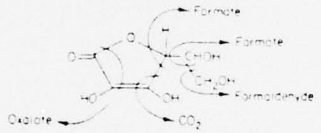
The periodate degradation of L-ascorbic acid yielded 1 mol of formaldehyde, 1 mol of oxalate, 1 mol of carbon dioxide, and 2 mol of formate, as shown in Table 1. The products and their amounts are consistent with known oxidation products of carbohydrates as described by Dryhurst (6).

TABLE I

PERIODATE DEGRADATION YIELD FROM 3.33 MMOL OF ABSCORBIC ACID

	Formaldehyde (mmol)	Oxalate (mmol)	Formate (mmol)	CO_2 (mmol)
Yield (mM)	3.12	3.36	5.15	3.30
Calculated yield (mM)	3.33	3.33	6.66	3.33
Percentage of Yield	93.5	100.8	77.0	99.0

However, the action of periodate on the enediol ring could not be predicted from classical periodate degradation. The data presented below show that cleavage of the molecule undoubtedly proceeds as follows:



Analysis of the degraded products gave chemical yields as shown in Table 1 and radioactivity yields as shown in Table 2. Total radioactive yields were generally 80–90% of the labeled ascorbic acid used in the reaction.

The dimedone assay for formaldehyde was quantitative, and 1 mol of the derivative was found per mol of ascorbic acid. This product from [1-^{14}C]ascorbic acid contained 96–99% of the recovered radioactivity. From [1-^{14}C]ascorbic acid only 1% of the carbon-14 was found in the dimedone precipitate. These results show clearly that the terminal glycol of the side chain is cleaved to form formaldehyde, and that this is a very clean reaction.

Titrametric oxalate determinations yielded an equivalent amount of oxalate based on the ascorbic acid used. The [6-^{14}C]ascorbic acid indicates that radioactivity contamination of this product was less than 1%. [1-^{14}C]Ascorbic acid degraded with periodate gave 82–87% of the total recovered radioactivity in oxalate and 12–17% as CO_2.

The possibility that the $^{14}CO_2$ in this degradation was derived from labeled dehydroascorbic acid, a common impurity in ascorbic acid, was investigated as follows. Labeled [1-^{14}C]ascorbic acid was oxidized with bromine to dehydroascorbic acid by adding 1.1 equivalents of Br_2, at which point the free bromine color persisted. This product was immediately subjected to periodate degradation. The results were essentially the same as with reduced ascorbic acid. This shows that dehydroascorbic acid was not the source of the $^{14}CO_2$. It also suggests that the mechanism of the periodate degradation of the enediol bond involves an initial oxidation

TABLE 2

DISTRIBUTION OF RADIOACTIVITY FROM PERIODATE DEGRADATION OF [1-^{14}C]ASCORBIC ACID, [6-^{14}C]ASCORBIC ACID, AND [1-^{14}C]DEHYDROASCORBIC ACID

	Recovery	Formaldehyde	Oxalate	Formate	CO_2
1.2 μCi As-6-^{14}C	μCi	1.018	0.006	0.022	0.01
in 3.33 mmol	Percentage[a]	96.4	0.61	2.04	0.94
1.2 μCi As-6-^{14}C	μCi	1.104	0.006	0.002	0.002
in 2.5 mmol	Percentage[a]	99.1	0.53	0.21	0.20
2.5 μCi As-1-^{14}C	μCi	0.001	1.91	0.005	0.259
in 2.5 mmol	Percentage[a]	0.03	87.8	0.24	11.9
2.5 μCi As-1-^{14}C	μCi	0.001	1.13	0.003	0.238
in 2.5 mmol	Percentage[a]	0.04	82.4	0.21	17.4
2.5 μCi dAs-1-^{14}C	μCi	0.001	1.83	0.003	0.244
in 2.5 mmol	Percentage[a]	0.05	88.4	0.14	10.8

[a] Calculated as percentage of total carbon-14 recovered.

of the ascorbate ring to the dehydroascorbic acid. The actual mechanism of formation of $^{14}CO_2$ from the C_1 carbon of ascorbate is not known. Under our conditions, oxalate is not oxidized to CO_2. It appears to be a minor pathway, probably involving a C_1–C_2 cleavage as an initial step.

Analysis for formic acid resulted in a calomel gravimetric yield of approximately 2 mol of formate per mol of ascorbic acid. The yield of formate is not quantitative in this distillation procedure, and this, not the efficiency of the periodate degradation itself, is considered the source of the low value. Both of the labeled ascorbic acids gave less than 1% of the initial radioactivity in the formate fraction. The efficiency of $^{14}CO_2$ recovery by sweep from the reaction vessel was tested and shown to be quantitative. One mole of CO_2 is produced per mol of ascorbic acid. The CO_2 from [6-^{14}C]ascorbic acid amounted to less than 1% of the radioactivity of the total sample. The CO_2 from [1-^{14}C]ascorbic acid, about 15%, is discussed earlier and seems to be a minor pathway in degradation of the enediol ring.

The amount of periodate consumed pH 7.5, under the condition used in this procedure, was not measured because a saturated solution of potassium metaperiodate was maintained during the reaction by an excess of the solid KIO_4. A total molar ratio of periodate to ascorbic acid of 8:1 was present in the initial reaction mixture. In earlier work, monitoring of the periodate degradation of ascorbic acid at pH 4.0 by titration with standard thiosulfate solution indicated that 5.5 mol periodate were consumed during the oxidation of 1 mol of ascorbic acid. The yield of formaldehyde, however, was less than 30% of that expected if one

equivalent of formaldehyde had been formed, possibly resulting from oxidation of the formaldehyde under acid conditions. Based on the reaction products, the amount of periodate consumed per mole of ascorbate is as follows. One mol of periodate is consumed during the cleavage of C_6 to formaldehyde, changing C_5 to an aldehyde. The enediol system of C_2-C_3 is cleaved using 2 mol of periodate to form oxalate from C_1 and C_2. This leaves C_3 in an acid form and a three carbon chain of 3-oxo-2-hydroxypropanoic acid from C_3, C_4, and C_5. C_5 is cleaved with 1 mol of periodate leaving C_3 and C_4 in the form of glyoxylate, which is rapidly degraded by the fifth mol of periodate to CO_2 from C_3 and formate from C_4.

SUMMARY

The degradation of ascorbic acid by periodate has been studied using [1-^{14}C] or [6-^{14}C]ascorbic acid, as well as quantitative yield of the various products. Ascorbic acid, oxidized at pH 7.5 is cleaved to give 2 mol formate, 1 mol CO_2, 1 mol of oxalate, and 1 mol formaldehyde. The oxalate and formaldehyde are derived from the 1- and 6-carbons, respectively. About 17% of C_1 is oxidized to CO_2 by a side reaction. Dehydroascorbic acid gives the same degradation products as ascorbic acid. Detailed procedures for the degradation are presented.

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Part IV B
Periodate Degradation of Ascorbic Acid
and Ascorbate Metabolites
by
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INTRODUCTION:

Many animal species, including man, are known to require ascorbic acid. Yet, the essential biological function of ascorbic acid remains unresolved. The biological function of ascorbic acid has been attributed to serving as an electron donor in electron transport (1), to affecting the concentration of cytochromes in the liver (2), to acting as a cofactor in the synthesis of hydroxyproline and hydroxylysine in collagen metabolism (3,4,5). In all of these systems other oxidation/reduction molecules such as glutathione can be shown to satisfy the role of ascorbic acid in *in vitro* systems. A specific biological function of ascorbic acid has not been determined.

Little is known about ascorbic acid metabolism. In the guinea pig, 60-70% of the ingested ascorbic acid is catabolized to carbon dioxide. The rat catabolizes 25% of its ingested ascorbic acid to CO_2 (6). The monkey has been shown to oxidize ascorbic acid to carbon dioxide when ascorbic acid is fed orally. When the monkey is given ascorbic acid i.v., no radioactive $^{14}CO_2$ is detected (7). Man has been found not to catabolize ascorbic acid to carbon dioxide after oral or intravenous injection (8,9). Thus, the oxidation of ascorbic acid to CO_2 may not be associated with an essential function of ascorbic acid in higher animals (10).

One of the best characterized metabolites of ascorbic acid is the formation of oxalate from C-2 and C-3 cleavage on

ascorbate. The use of C-1 and C-2 double labeled ascorbic acid experiments have shown that oxalate comes from carbons 1 and 2 of ascorbate (11). Excretion of oxalate is 4-10% of the catabolic products and persists during deprivation of ascorbic acid in man (12). However, the four carbon intermediate, believed to be threonate, after the cleavage between C-2 and C-3 does not appear to be catabolized further (13). Additional metabolites have been found since the discovery of oxalate. Ascorbate-2-sulfate (ascorbate sulfate) was initially discovered in brine shrimp cysts by Mead and Finamore (14). Ascorbate-2-sulfate has been found as a urinary and soft tissue metabolite in all animals examined, including man (15,16). Ascorbate sulfate is a minor urinary metabolite comprising about 5% of the total urinary ascorbate metabolites in the monkey (17). A 2-O-methyl L-ascorbate derivative has been found in the rat (18). All of these known ascorbate metabolites comprise only a small number of the total ascorbate metabolites. Major ascorbate metabolites are believed to be catabolized from dehydroascorbic acid. However, no ascorbic acid oxidase has been reported in animals. The structures of the major ascorbate metabolites have not been determined.

Investigations of ascorbic acid metabolites have generally been concerned with modifications on the enediol ring. Studies on urinary ascorbate metabolites, using column chromatography,

suggest a large number of undetermined ascorbate metabolites exist (17). These column chromatographs of ascorbic-1-^{14}C and ascorbic-6-^{14}C give a similar pattern of metabolites, and it is believed that in most metabolites, the ascorbic acid carbon skeleton remains intact (17). The number of these ascorbate metabolites are difficult to rationalize without postulating side chain metabolism. Recently, Hornig has proposed a 6-carboxy derivative of ascorbate sulfate as a possible metabolite of ascorbate sulfate (19). Tolbert has also proposed carbon-6 oxidation as metabolites of ascorbic acid metabolism (10). In addition, studies of ascorbic acid precursors in plants have led F. A. Loewus to propose a biosynthetic pathway of ascorbic acid which requires epimerization of carbon 5 (20).

Experiments were designed to examine the side chain metabolism of ascorbic acid using periodate oxidations. Periodate oxidations have been a favorite analytical technique for many carbohydrate chemists. Periodate will cleave 1,2-diols, 1,2-aminoalcohols, and keto compounds in a predictable manner and can be used to detect derivatization. This thesis contains a chemical study of periodate oxidation on ascorbic acid. Periodate oxidation of labeled ascorbic acid has been developed to establish, from degradation product analysis, the mechanism of periodate on ascorbic

acid. This periodate degradation technique was applied to labeled urinary ascorbate metabolites from monkeys and rats given ascorbic-6-^{14}C acid.

PART I

PERIODATE DEGRADATION OF ASCORBIC ACID: A CHEMICAL STUDY

Periodate oxidations have become a classical analytical tool in carbohydrate chemistry. Periodate research has provided chemists with conditions favorable for periodate to selectively oxidize 1,2-diols, 1,2-aminoalcohols, 1,2-hydroxyaldehydes and ketones, 1,2-diketones, and 1,2-amino-aldehydes. Some active methylene groups have been known to oxidize with periodate. The action of periodate on these compounds has been well studied (21). Periodate degradation products from these compounds can be accurately predicted.

Periodate has been shown to rapidly attack the sulfur atom of methionine and cystine (22). However, the oxidation of sulfur containing compounds is not general. Further study on the periodate oxidation of sulfur containing compounds must be made to understand the reactions and mechanisms (21).

The predictable mechanism of periodate oxidation was used to suggest the areas of periodate attack on ascorbic acid. From the periodate degradation products described by Dryhurst (21), ascorbic acid was suggested to cleave the diol side chain between carbons 5 and 6. Periodate was anticipated to oxidize the enediol lactone ring between carbons 2 and 3 but, because this is an enediol and a very

labile ring when partly oxidized, the nature and amount of products could not be predicted. Further periodate degradation was considered a possibility between carbons 3 and 4, and 4 and 5 after partial degradation.

Recently, Verma and Grover have used periodate oxidation of the C-6 carbon to formaldehyde followed by gravimetric assay of the dimedone-formaldehyde derivative as the basis of an assay for ascorbic acid however, no experimental procedures were included (23). Horowitz and King (24) developed a degradation procedure for the osazone of labeled ascorbic acid which has been used by Loewus (20). Periodate oxidation of ascorbic acid has been studied by Juni and Heym who published results showing that glyoxalate was formed in 91% yield (25). This result is in disagreement with classical predictions.

The work of Horowitz and King did not establish definitively that observed products come from the C-1 and the C-6 carbons of the osazone and further, the osazone could degrade in a different way from ascorbic acid itself. Since previous periodate oxidation studies on ascorbic acid were not in agreement, the establishment of periodate degradation products from ascorbic acid was necessary as a first step in this study.

Materials: Ascorbic-1-^{14}C acid was purchased from New England Nuclear Corporation. Ascorbic-6-^{14}C acid was

synthesized as described (26). Ascorbate-6-^{14}C sulfate was synthesized by Dr. B. M. Tolbert's laboratory as described (27). Sodium carbonate-^{14}C solution was prepared from standardized barium carbonate-^{14}C. Potassium metaperiodate, 5,5-dimethyl-1,3-cyclohexanedione (dimedone) and ascorbic acid were reagent grade materials.

Periodate Oxidation: A three necked 250 ml. round bottom flask was charged with 100 ml. of 0.11 M. KIO_4, pH 8.1. The flask was fitted with a pH electrode, a 60 ml. pressure compensated dropping funnel, a sweep gas inlet tube extending to the bottom of the flask, and an exit tube leading to two 15 ml. CO_2 absorption traps and a stirring bar. The reaction vessel was covered with dark paper to exclude light and left at room temperature. The CO_2 traps were filled with 2-amino-ethanol (ethanolamine). A 2.5 mM sample of L-ascorbic acid was added from the funnel by drops over fifteen minutes. The reaction mixture was stirred for one hour. The pH ranged from 7.5 to 8.1 with 5 M KOH. Five ml. of concentrated HCl was added to the reaction mixture and then 15 ml. of 6 M. sodium arsenite was added by drops over ten minutes. After the yellow color disappeared, a nitrogen sweep of the vessel at a rate of 2 bubbles/second was maintained for one hour.

Formaldehyde Determination: The reaction mixture was diluted with water and enough 5 M. KOH was added to bring the final solution to pH 4.6 in 400 ml. A 20 ml. aliquot was taken from

the solution and 2 ml. of 5,5-dimethyl-1,3-cyclohexanedione (dimedone) solution (80 mg./ml. 95% ethanol) was added. The solution stood overnight. The white precipitate was collected on a tared sintered glass filter, dried, and weighed. The melting point of the dimedone derivative was 191-192.5°C.

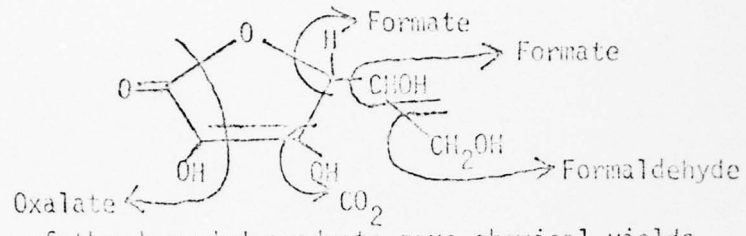
Oxalate Determination: The formaldehyde filtrate was neutralized with 5 M. KOH and 4 ml. of 0.5 M. calcium acetate was added. The solution stood for one hour. The calcium oxalate precipitate was collected on a fine sintered glass filter and washed with 5 ml. of water. The filtrate was removed for the formic acid determination. The precipitate was washed with 2 ml. of 4 M. acetic acid solution and the filtrate was discarded. The remaining precipitate was treated with 6 ml. of 2 M. HCl, filtered, and washed with 2 ml. water. One ml. of this solution was used for radioassay. The remaining solution was heated to 60° and the oxalic acid was determined by titration with 10^{-3} M. standard potassium permanganate solution using a pink endpoint.

Formic Acid Determination: The oxalate filtrate was vacuum distilled to dryness at 34°C. To the residue, 10 ml. of water was added, followed by 10% phosphoric acid added by drops until the solution reached a pH of 2. The solution was distilled to dryness in vacuo. Ten ml. of water was added to the residue and the solution was distilled to dryness. The last step was repeated once.

The four distillates were combined and neutralized with 0.1 M. KOH. The neutral solution was concentrated in vacuo to 3 ml. and transferred with 15 ml. wash water to a large test tube. Three ml. of 7% mercuric chloride, 1 ml. of saturated sodium acetate, and 0.2 ml. of 4 M. HCl were added. The tube was closed with a stopper containing an N_2 sweep inlet tube and an exit tube leading to two 15 ml. CO_2 absorption traps filled with ethanolamine. The test tube was covered with black tape and heated in a boiling water bath for one hour with an N_2 sweep of about 2 bubbles/second. The test tube was cooled in an ice bath for thirty minutes. The calomel precipitate was collected, air dried on a tared glass filter and weighed. The reaction of mercuric chloride with formic acid to form mercurous chloride liberates carbon dioxide as shown:

$$2\ HgCl_2 + HCOOH \longrightarrow Hg_2Cl_2 + 2\ HCl + CO_2$$

Results and Discussion: The periodate degradation of L-ascorbic acid yielded one mole of formaldehyde, one mole of oxalate, one mole of carbon dioxide, and two moles of formate as shown in Table I. The products and their amounts are consistent with known oxidation products of carbohydrates as described by Dryhurst (21). However, the action of periodate on the enediol could not be predicted from classical periodate degradation. These data show that cleavage of the molecule undoubtedly proceeds as follows:



Analysis of the degraded products gave chemical yields as shown in Table I and radioactivity yields as shown in Table II. Total radioactive yields were generally 80-90% of the labeled ascorbic acid used in the reaction. The dimedone assay for formaldehyde was quantitative, and one mole of the derivative was found per mole of ascorbic acid. The product from ascorbic-6-^{14}C acid contained 96-99% of the recovered radioactivity. From ascorbic-1-^{14}C acid, only 1% of the carbon-14 was found in the dimedone precipitate. The results clearly show that the terminal glycol of the side chain is cleaved to form formaldehyde and this particular side chain oxidation is a very clean one.

Titrametric oxalate determinations yielded an equivalent amount of oxalate based on the ascorbic acid used. The ascorbic-6-^{14}C acid indicates that radioactivity contamination of this product was less than 1%. Ascorbic-1-^{14}C acid degradation gave 82-87% of the total recovered radioactivity in oxalate and 12-17% as CO_2.

The possibility that the $^{14}CO_2$ in this degradation was derived from labeled dehydroascorbic acid, a common impurity

in ascorbic acid, was investigated as follows. Labeled ascorbic-1-^{14}C acid was oxidized with bromine to dehydroascorbic acid by adding 1.1 equivalents of Br_2 at which point the free bromine color persisted. The product was immediately subjected to periodate degradation. The results were essentially the same as with reduced ascorbic acid. This shows that dehydroascorbic acid was not the $^{14}CO_2$ source. It also suggests that the periodate degradation mechanism on the enediol bond involves an initial oxidation of the ascorbate ring to the dehydroascorbic acid. The actual mechanism of formation of $^{14}CO_2$ from the C-1 carbon of ascorbate is not known. Under the reaction conditions, oxalate is not oxidized to CO_2. It appears to be a minor pathway probably resulting from C_1-C_2 cleavage as the initial step.

Analysis for formic acid resulted in a calomel gravimetric yield of approximately two moles of formate per mole of ascorbic acid. The yield is not quantitative in the distillation procedure and this, not the efficiency of the periodate degradation itself is considered the source of the low value. Both of the labeled ascorbic acids gave less than 1% of the initial radioactivity in the formate fraction. The efficiency of the $^{14}CO_2$ absorption traps was tested using barium-^{14}C-carbonate and shown to be quantitative.

The carbon dioxide from ascorbic-6-^{14}C acid amounted to less than 1% of the radioactivity of the total sample. The CO_2 from ascorbic-1-^{14}C acid, about 15%, is discussed earlier and appears to be a minor pathway in the degradation of the enediol lactone ring.

Ascorbate-6-^{14}C sulfate was degraded with periodate. Table II shows that the results are the same for the degraded products as found with ascorbic-6-^{14}C acid. This indicates that side chain oxidation with periodate does not appear to be affected by derivatization on the enediol lactone ring.

The amount of periodate consumed at pH 7.5 under the conditions described in the experiment was not measured because a saturated solution of the potassium metaperiodate was maintained during the reaction by an excess of the KIO_4. A total molar ratio of periodate to ascorbic acid of 8:1 was present in the initial reaction mixture. In earlier work, monitoring of the periodate degradations of ascorbic acid at pH 4.0 by titration with standard thiosulfate solution indicated 5.5 moles of periodate were consumed during the oxidation of one mole of ascorbic acid. The yield of formaldehyde, however, was less than 30% of that expected if one equivalent of formaldehyde had been formed, possibly resulting from oxidation of the formaldehyde under acid conditions. Based on the reaction products, the amount of periodate consumed per mole of ascorbate is as follows: One mole of periodate is

consumed during the cleavage of C-6 to formaldehyde changing C-5 to an aldehyde. The enediol system of C-2 and C-3 is cleaved using two moles of periodate to form oxalate from C-1 and C-2. This leaves C-3 in an acid form and a three carbon chain of 3-oxo-2-hydroxy-propanoic acid from C-3, C-4, and C-5. C-5 is cleaved with one mole of periodate leaving C-3 and C-4 in the form of glyoxalate which is rapidly degraded by the fifth mole of periodate to CO_2 from C-3 and formate from C-4.

PART II

CHEMICAL NATURE OF URINARY METABOLITES OF ACORBIC ACID IN RATS:

A rat was given ascorbic-6-^{14}C acid and the urinary ascorbate metabolites were examined by periodate degradation. Determination of the nature of the ascorbate side chain was the primary interest. Periodate oxidation of ascorbic acid and all metabolites with an intact -CHOH-$^{14}CH_2OH$ should yield formaldehyde-^{14}C, as demonstrated in part I of this thesis. If the side chain is chemically altered, either by derivatization or oxidation/reduction, it will not yield labeled formaldehyde. The urinary ascorbate metabolites from the labeled rat were degraded with periodate. These urinary ascorbate metabolites were also partly separated by column chromatography and the separated peaks were periodate degraded.

Animal Procedures: A 330 g. Long-Evans Hooded rat was given subcutaneously 33.2 μCuries, 10.2 mg. of ascorbic-6-^{14}C acid in 0.75 ml. of sterile saline. The rat was fed a normal diet and its daily urine excretion was collected and filtered with washing through Whatman #42 filter paper using a Buchner funnel to separate the feces. The total volume of urine and washings was measured and one ml. was used for radioactivity assay by a liquid scintillation counter. The total carbon-14 per day was determined and the remaining urine was frozen and stored for later analysis.

Periodate Degradation of Whole Urine: Five to eight ml. aliquots of the daily urine samples were ultrafiltered using a UM-2 diaflow membrane (excludes greater than 2000 MM) in an Amicon stirring cell under a nitrogen pressure of 48 p.s.i. A one ml. sample was assayed for radioactivity. 2.5 mM. of L-ascorbic acid was added to the urine sample as a carrier and the solution was degraded with periodate as described in part I. After the quenching of the reaction mixture and the completion of the N_2 sweep, as described in part I, the solution was brought to a total volume of 400 ml. at pH 4.6. The solution was gravity filtered through Whatman #1 filter paper and a 20 ml. aliquot was removed. Two ml. of dimedone solution was added to the aliquot and the solution was allowed to stand overnight. The dimedone-formaldehyde precipitate was collected on a sintered glass filter as described in part I. The precipitate was dried and assayed for radioactivity with a liquid scintillation counter. The radioactivity recovered in the dimedone-formaldehyde precipitate was compared to the radioactivity of the urine sample.

DEAE-32 Column Chromatography: Four to six ml. aliquots were ultrafiltered through a UM-2 membrane using an Amicon stirring cell under 48 p.s.i. of nitrogen. The filtered urine samples were concentrated under a stream of nitrogen to 1.5 ml. at 37°C. A DEAE-32 column (1 x 30 cm.)

was prepared and a pressure head was pregenerated on the column until a stable flow rate of 0.75 ml./min. was maintained. The sample was layered on the column and gradient I (0.006-0.009 M. H_2SO_4, 75 ml. each) was applied immediately after the sample. The column effluent was run through the flow cell of an Isco UA-4 absorbance monitor set at 254 nanometers. Five minute collections were generally made during the running of gradient I and ten minute collections were made when the additional gradients were applied. At the end of gradient I, 0.009 M. H_2SO_4 was applied to the column until an ascorbate-2-sulfate peak was eluted.

A Strip gradient (0.009 M. H_2SO_4 - 0.02 M H_2SO_4 plus 0.2 M Na_2SO_4, 50 ml. each) followed and this gradient eluted the remaining radioactive peaks. One ml. from each fraction was aliquoted and assayed for radioactivity. The remaining eluent in each fraction was neutralized with 0.5 M. KOH and stored frozen at -20°C. The radioactivity assay of each fraction was graphed as the DPM per fraction versus fraction number on semi-log paper.

Periodate Degradation of Peaks: The fractions containing radioactivity peaks were pooled and one ml. of the pooled fraction was assayed for radioactivity. The remainder of the peak was used for periodate degradation, first 440 mg. of L-ascorbic acid was added. The peak was treated with 0.11 M. saturated solution of potassium periodate

under the conditions described in part I. After quenching of the reaction and a nitrogen sweep for one hour, the solution was brought to pH 4.6 in a total volume of 400 ml. A 20 ml. aliquot was taken and analyzed for the degradation products described in part I.

Results: A graph of the total carbon-14 excreted in the urine per day versus time for the rat is shown in Figure I. The half-life for the ascorbic-6-^{14}C in the rat was 2.0 days. This result is consistent with previously determined half-lifes for ascorbic acid in rats.

The results of periodate degraded whole urine samples from days 2, 9, 18, and 23 are reported in Table III. These results are given as the percentage of radioactivity recovered in the dimedone-formaldehyde precipitate compared to the total radioactivity of the urine sample.

A DEAE-32 column chromatograph of day one rat urine is shown in Figure II. The strip peak fractions, which appear after application of the Strip gradient, were pooled and degraded with periodate. Analysis for radioactivity of the degraded products is given in Table IV. A second DEAE-32 column chromatograph of day one rat urine is shown in Figure III. The peak designated as peak II was pooled and treated with periodate. The distribution of radioactivity among the products is reported in Table IV. DEAE-32 column chromatography of day three rat urine was made using a

modified gradient I. Gradient I (0.000 to 0.009 M. H_2SO_4, 75 ml. each) was used to improve resolution on the region containing the weak acids. The elution pattern (Figure IV) resulted in separation of some of the weak acid compounds. The neutral peak (fractions 4 and 5) was treated with periodate. Analysis of periodate degraded products from the neutral peak are reported in Table IV. The weak acid peak (fraction 9 through 14) was degraded with periodate and radioactivity analysis of the products is given in Table IV. A final DEAE-32 column chromatograph of day two urine (Figure V) resolved six peaks of radioactivity. The neutral peak, the weak acid peak, peak I (fractions 21-25), and peak II were pooled separately and periodate degraded. The analysis of the degraded products from these peaks is given in Table V.

PART III

CHEMICAL NATURE OF URINARY METABOLITES OF ASCORBIC ACID IN THE MONKEY:

An experiment analogous to the labeling of the rat was made on a monkey in a cooperative study with the Letterman Army Institute of Research, Presidio of San Francisco. Experimental results from monkeys are particularly interesting because they can be extrapolated to man. The ascorbic acid requirement for primates has been shown to be analogous to man's requirement (7). Primate ascorbic acid metabolism appears to be similar to the metabolism of man. Both monkey and man have been found to maintain a pool of ascorbate and the turnover time for ascorbic acid in both species is similar.

Experimental Procedure: A 3 kg. macaque monkey was given i.v. 65.3 μCuries, 25 mg. of ascorbic-6-^{14}C acid and the twenty-four hour urines were collected, assayed for radioactivity and frozen. The monkey was fed a commercial monkey chow diet ad lib which supplied about 300 mg. of ascorbic acid per day. Two-thirds of each urine was shipped frozen in dry ice from Letterman Army Institute of Research to the University of Colorado, Boulder laboratory and stored in a deep freeze until analysis was made. The urines were assayed for carbon-14 radioactivity and the twenty-four hour excretions of carbon-14 were determined.

Periodate Degradation of Whole Urines: Five to eight ml. samples of urine were prepared for periodate degradation as described in part II. The samples were degraded with periodate as described in part I. Following termination of periodate degradation and collection of carbon dioxide, the solution was brought to a total volume of 400 ml. at pH 4.6. A 20 ml. aliquot was removed and 2 ml. of the dimedone solution (described in part I) was added. The dimedone-formaldehyde precipitate was collected and assayed for radioactivity.

DEAE-32 Column Chromatography: A five ml. aliquot of day one monkey urine was prepared by the method described in part II. The urine sample was concentrated to one ml. under a stream of nitrogen at 37°C. The one ml. of sample was layered on a DEAE-32 column (1 x 30 cm.) and the resolution of ascorbate metabolites was made using the gradients described in part II. One ml. aliquots of the collected fractions were assayed for radioactovity and the remaining eluents were neutralized by the method indicated in part II. The DPM per fraction versus fraction number was graphed on semi-log paper.

Periodate Degradation of Peaks: The fractions containing radioactivity peaks were pooled and treated as described in part II. 440 mg. of L-ascorbic acid was added to the pooled peak prior to periodate degradation as described in part II.

Results: A graph of the total carbon-14 per day versus time is shown in Figure VI. The turnover time for the ascorbic-6-^{14}C in the monkey was 20 days.

Whole urine samples from twenty-four hour collections of days 1, 6, 29, 39, 41, 43, and 57 were degraded with periodate. The percentage of recovered radioactivity in the dimedone-formaldehyde precipitate to the sample radioactivity is given in Table VI.

The DEAE-32 column chromatography of day one monkey urine is given in Figure VII. The neutral peak (fractions 8-10), the weak acid peak (fractions 14-21), the ascorbate sulfate peak (fractions 45-47), and a pooled strip peak were degrade with periodate. The results of the degraded products carbon-14 analysis are reported in Table VII.

PART IV

DISCUSSION OF RESULTS:

Baker has shown that man does not catabolize ascorbate-1-^{14}C to $^{14}CO_2$ (8,9). A similar study on macaque monkeys has shown that when monkeys were orally administered ascorbic-1-^{14}C, ascorbate-1-^{14}C sulfate, and ascorbic-6-^{14}C, they released significant amounts of $^{14}CO_2$ in their breath (7). When ascorbic-1-^{14}C, ascorbate-1-^{14}C sulfate, and ascorbic-6-^{14}C were given i.v. to the monkeys, no radioactive carbon dioxide was detected in their breath. The feces from the monkeys contained negligible radioactivity (20). In both man and monkeys given i.v. ascorbic-^{14}C the urine is the major excretion path and contains the major ascorbic acid metabolites.

Periodate degradation of rat and monkey whole urines (Tables III and VI) show 55% of the ascorbate urinary metabolites retain the primary hydroxyl group at carbon 6. The 55% recovery of formaldehyde-^{14}C suggests ascorbate side chain modification for the remaining 45%. The modified structures of the ascorbate side chain cannot be elucidated from the degradation product analysis used with periodate. Periodate degradation of the ascorbate side chain can be used effectively, if the side chain structure is a diol or keto derivative. Periodate will also degrade amine or thio derivatives in a manner analogous to the

oxygen derivatives (21). If the ascorbate side chain has been esterified, alkoxy substituted, or reduced, periodate will not degrade the side chain. From the data in Tables III and VI, possible ascorbate metaholites could include reduction at either carbons 5 or 6 or both, alkoxy substitution on carbons 5 or 6 would not allow periodate to cleave the ascorbate side chain. The side chain could be esterified. Carbon 6 could form the acid or aldehyde or deoxy derivative. Also, amine and thio derivatization could be possible structural modifications on the ascorbate side chain that would not degrade with periodate. Further possible side chain modifications include reduction of carbon 5. All of these ascorbate side chain derivatives could result in 55% recovery of formaldehyde-^{14}C when the urines are treated with periodate.

A DEAE-32 cellulose column was used to resolve six major radioactive peaks from rat urine and four major radioactive ascorbate peaks from monkey urine (Figures V and VII). They are called in order of elution: neutral peak, weak acid peak, peak I, peak II, ascorbate sulfate peak, and strip peak I. The peaks were separated using dilute sulfuric acid gradients as described in parts II and III. It is not known whether these peaks represent single compounds or mixtures of labeled products but, the number, shape and limited resolving power of the column would suggest that many of them are

mixtures of compounds.

The resolution of four major radioactive ascorbate peaks from the monkey urine conflicts with the results of Knight (17). Knight had shown in the monkey that six major radioactive ascorbate peaks were separated from the urine of a monkey given either ascorbic-1-^{14}C or ascorbic-6-^{14}C (17). This suggested an intact ascorbate structure for all the major metabolites. In this study only four major peaks were in urine from ascorbic-6-^{14}C acid, see Figure VII. Peaks I and II are not present. A careful examination of the experimental conditions suggest an explanation. At the time of Knight's work, the long turnover time of ascorbic acid in the monkey was not known. In Knight's experiment, the monkey used had been given ascorbic-1-^{14}C acid only sixty days prior to the i.v. administration of ascorbic-6-^{14}C. The ascorbic acid turnover time in the monkey is twenty days. This permits only three half-lifes prior to the administration of the ascorbic-6-^{14}C. Peaks I and II observed by Knight are believed to be a result of ascorbate-1-^{14}C metabolite contamination. The current separation suggests peaks I and II lose the C-6 carbon suggesting ascorbate side chain oxidation and decarboxylation in the monkey.

The rat did not show this loss of peaks I and II. The rat urine was usually separated into six major radioactive

ascorbate peaks. Each of these peaks were pooled and degraded with periodate as described in part II. The analysis of the degradation products is shown in Tables IV and V. Two general observations are immediately available; first, each of these peaks from periodate degradations are mixtures of compounds, and second, the recovered radioactivity is not quantitative, which suggests other degradation products containing the carbon-14 label are present.

The degradation of the neutral peak (Tables IV and V, Figures IV and V) analyzed as 69% of the radioactivity as formaldehyde-^{14}C, indicating that much of the primary hydroxyl group remains on the ascorbate C-6 carbon. The radioactivity recovery of oxalate-^{14}C is indicative of a mixture of compounds in this peak. Comparison of the elution volumes for ascorbic acid with the neutral peak on the DEAE-32 column indicates ascorbic acid is not found in the neutral peak. The oxalate-^{14}C and lack of quantitative radioactivity recovery data suggest several compounds in the neutral peak are side chain modified. The neutral peak's major componenents appear to contain an unmodified side chain. These major components then must be modified on the enediol lactone ring.

The weak acid peak (Figures IV and V) was degraded with periodate. The formaldehyde-^{14}C recovery was 52 and 56% of the total (Tables IV and V). The radioactive analysis of oxalate,

formate, and carbon dioxide did not recover all of the radioactivity found in the solution. Only 5% more of the total carbon-14 was found in these three degradation products. The structures of the compounds in this peak are believed to contain ascorbate side chain modifications, ascorbate enediol lactone ring derivatizations, and excess ascorbic acid.

Peak I from the rat urine DEAE-32 column (Figure V, Table V) was degraded with periodate and resulted in a quantitative recovery of formaldehyde-^{14}C. The ascorbate side chain is intact and modifications are probably on the ascorbate enediol lactone ring.

Peak II (Figures III and V) gave anomalous results when periodate degraded. In Figure III, the day one urine resolution followed with periodate degradation of peak II recovered 14% of the radioactivity as formaldehyde-^{14}C (Table IV). The day two urine separation and periodate treatment (Figure V, Table V) recovered 71% of the radioactivity as formaldehyde-^{14}C. The reason for this inconsistency is not known. Peak II is known to vary in shape and magnitude and perhaps different ascorbate metabolites are present.

The strip peak (Figure II, Table IV) was degraded with periodate and gave as products; 20% formaldehyde-^{14}C, 71% oxalate-^{14}C, and 9% formate-^{14}C. The strip peak could

actually contain more compounds than the other peaks, since there is little resolution at this stage. The oxalate-^{14}C and formate-^{14}C are indicative of an ascorbate side chain oxidative process. Oxalate is a known ascorbate metabolite from carbons 1 and 2 (11), but since this is labeled oxalate, it must come from carbons 5 and 6. The recovery of oxalate-^{14}C from periodate degradation of the urine of a rat given ascorbic-6-^{14}C indicates both carbons 5 and 6 of ascorbate have been oxidized. Periodate will cleave carbons 5 and 6 of ascorbate as oxalate only if an α-ketoacid has been formed on the ascorbate side chain. The formate-^{14}C recovery could suggest carbon 6 has been oxidized to the aldehyde.

The resolution of the radioactive ascorbate peaks from rat urine by column chromatography and ascorbate side chain analysis of these peaks suggests the ascorbate side chain is modified in some of the metabolites. From the data in Tables IV and V, several ascorbate side chain modifications can be proposed and their degradation with periodate can be predicted:

Ascorbate Side Chain	Periodate Degradation Product
$-CHOH-{}^{14}CH_2OH$	$H{}^{14}CHO$
$-\overset{O}{\overset{\|}{C}}-{}^{14}CH_2OH$	$H{}^{14}CHO$
$-CHOH-{}^{14}CHO$	$H{}^{14}COOH$
$-\overset{O}{\overset{\|}{C}}-{}^{14}COOH$	$^{-}OOC-{}^{14}COO^{-}$
$-CHOH-{}^{14}COOH$	$OHC-{}^{14}COO^{-}$

Although the last periodate degradation product, glyoxalate, has not been conclusively demonstrated, preliminary work has shown a radioactive periodate degradation product which is believed to be glyoxalate. Glyoxalate can be obtained from the ascorbate side chain if carbon 6 has been oxidized to the acid with carbon 5 remaining intact. Periodate will not cleave α-hydroxyacids and thus glyoxalate is the periodate degradation product.

The DEAE-32 column separation of the macaque monkey urine samples was discussed earlier. Periodate degradation of the neutral peak (Figure VII, Table VII) gave a

48% formaldehyde-^{14}C recovery. The other analyzed products showed no radioactivity. This indicates the ascorbate side chain is modified and periodate will cleave the side chain in a manner that present analysis will not isolate the radioactive degradation product. The incomplete recovery of carbon-14 indicates the neutral peak is a mixture of at least two compounds.

The weak acid peak from the monkey gave a 70% formaldehyde-^{14}C recovery (Table VII). The recovery is not quantitative and implies that the weak acid peak also contains a mixture of compounds. The neutral and weak acid peaks contain the major ascorbate metabolites of the monkey. These periodate results seem to be in agreement with periodate degradations on the whole urine. The absence of peaks I and II in the monkey indicates loss of the C-6 carbon as mentioned earlier in these metabolites.

The ascorbate sulfate peak was treated with periodate. The results from the degradation of this peak indicate the ascorbate sulfate peak is not a single compound. The oxalate-^{14}C recovery from degradation with periodate suggests the presence of an ascorbate side chain metabolite containing an α-ketoacid (Table VII). This oxalate-^{14}C recovery is analogous to periodate degradation of the strip peak from the rat.

The strip peak from the monkey urine degrades as

formaldehyde-^{14}C (Table VII), which indicates the ascorbate side chain is not modified.

Periodate degradations of the DEAE-32 column peaks indicate some of the ascorbate metabolites contain side chain modifications which are not analyzed by the present methods. The oxalate-^{14}C recovery suggests that an α-ketoacid ascorbate side chain is a catabolic product of ascorbate metabolism. The structures of the major ascorbate metabolites have not been elucidated.

The data from periodate degradations of whole urines and DEAE-32 column resolved peaks suggest the ascorbate side chain is extensively modified during catabolism. It cannot be determined from periodate degradation data alone if the ascorbate side chain is in part reduced as well as oxidized. A recent study of ascorbate urinary metabolites from a monkey given i.v. ascorbic-6-^{3}H has provided data which indicates the ascorbate side chain is oxidized during catabolism (29). The labeling experiment (macaque monkey i.v., 253 μCi., 50 mg. ascorbic-6-^{3}H) has shown that 43% of the tritium is excreted as water. Twenty-four hour urine samples were collected from the ascorbic-6-^{3}H labeled monkey. The tritium found as $^{3}H_2O$ was measured in addition to the total urine radioactivity and the ascorbate metabolites. The rate of $^{3}H_2O$ formation is equivalent to the rate of organic ascorbate metabolite excretion. The $^{3}H_2O$ was shown

to turnover in twenty days, which is the same turnover time found for ascorbic acid. The body water turnover time in the monkey has been measured to be seven days. If the $^{3}H_2O$ measured in this experiment turned over in seven days, then the release of tritium would correspond to the body water pool and fail to show ascorbate carbon 6 oxidation. The 43% recovery of $^{3}H_2O$ and twenty day half-life is highly suggestive of ascorbate side chain oxidation.

From this data, the 6-deoxyascorbic acid can be eliminated as an ascorbate urinary metabolite. The loss of the tritium would not occur if the ascorbate side chain was being reduced. However, the data cannot eliminate carbon 5 and/or 6 esterification as possible ascorbate side chain metabolites.

Periodate degradation of the whole monkey urines (Table VI) shows ascorbate side chain oxidation to be constant with time. This constant rate of ascorbate side chain oxidation may be a result of the monkey's inability to synthesize ascorbic acid and its ascorbate storage system. Periodate degradation of the whole rat urine (Table III) shows ascorbate side chain oxidation increases with time. This increase in ascorbate side chain oxidation could be a result of synthesis of ascorbic acid by the rat. Present data indicate the rat and monkey should have a

circulating ascorbic acid pool primarily located in the blood and a long turnover ascorbate pool. If this circulating ascorbate pool slowly exchanges with the long turnover ascorbate pool, the rate of labeling this slow exchanging ascorbate pool with ascorbic-6-^{14}C would be equal to the rate of ascorbate depletion from this pool. In the case of the rat, this slow exchanging pool will not be saturated with ascorbic-6-^{14}C until the synthesized ascorbate has been catabolized. The rate of ascorbate side chain oxidation would appear to increase with time.

Prior to the i.v. labeling of the macaque monkey with ascorbic-6-^{14}C acid, the monkey fasted for twelve hours. The monkey also fasted twenty-four hours post-label. This procedure allows the labeled ascorbic acid to enter the ascorbate pool with the long turnover time. This long turnover ascorbate pool appears to be in the reduced ascorbate side chain conformation. The ascorbate side chain oxidized derivatives are formed and released from this pool which slowly exchanges with the circulating pool.

The 55% formaldehyde-^{14}C, oxalate-^{14}C, and formate-^{14}C recoveries from periodate degradations, and the recovery of 43% $^{3}H_2O$ from the ascorbic-6-^{3}H study imply that the ascorbate side chain is oxidized. This data can be used to suggest

possible ascorbate side chain derivatives with the common factor of tritium loss on the ascorbate C-6 carbon. The possible structures include:

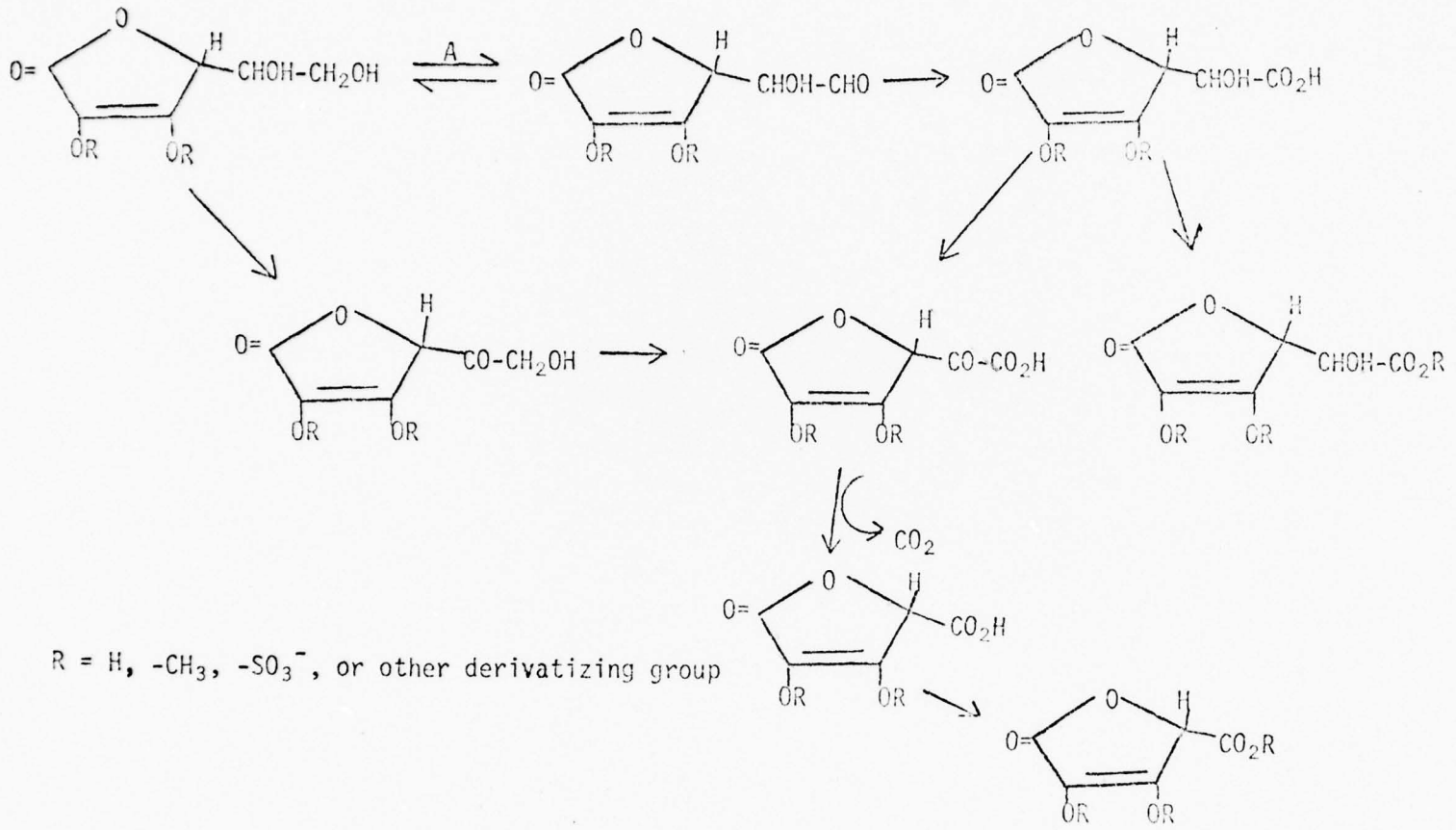
-CHOH-CHO

-CHOH-COOH or -CHOH-COOR

$$\text{-}\overset{\overset{O}{\|}}{C}\text{-COOH} \quad \text{or} \quad \text{-}\overset{\overset{O}{\|}}{C}\text{-COOR}$$

Analogous thio or nitrogen derivatizations are possible but, seem less likely from a metabolism perspective.

The data further suggest the ascorbic acid C-6 carbon is actively metabolized by a process that loses hydrogen. It is apparent that the nature of ascorbate carbons 5 and/or 6, to periodate degradations, changes in the ascorbate metabolites. The limited number of different types of biochemical reactions and on the basis of the data allow the postulation of a reasonable metabolic pathway. A speculative metabolic scheme for ascorbate catabolism is as follows:



R = H, $-CH_3$, $-SO_3^-$, or other derivatizing group

Pathway A is characteristic of known catecholamine processes, particularly norepinephrine. Norepinephrine is side chain oxidized by monoamine oxidase to the aldehyde. The aldehyde is either oxidized or reduced to the acid or alcohol. The structural nature of ascorbic acid and the location of ascorbic acid in the brain permit the speculation of an analogous oxidative process for the ascorbate side chain. Harold Stuber has synthesized the L-saccharoascorbic acid and has preliminary evidence, through column and thin layer chromatography, to suggest this C-6 acid derivative is an ascorbic acid metabolite. The reversibility of the first step in the proposed ascorbate metabolic pathways is not proven. The loss of tritium during oxidation is known. The major reason for proposing the reversibility of the oxidation to the aldehyde is by analogy to catechol metabolism.

The recovery of oxalate-^{14}C indicates the oxidation of the ascorbate carbon 5 is a probable metabolite. Loewus has suggested the importance of carbon 5 oxidation in the biosynthesis of ascorbic acid (20). The 5-ketoacid compound could be oxidized at the C-6 carbon and form the α-ketoacid. The ascorbate ketoacid derivative would give oxalate when treated with periodate. The ascorbate ketoacid could be easily decarboxylated and form the five carbon compounds suggested by loss of peaks I and II from the DEAE-32

cellulose cloumn resolution of the monkey urine.

The formation of a series of 5-deoxy catabolites is possible from the experimental results in this thesis. It does not seem probable on the basis of intermediate metabolism. In addition to these speculative ascorbate metabolic pathways, analogous thio and nitrogen derivatizations can be proposed.

Another interest in C-6 oxidized derivatives of ascorbate would arise from the ability of these functional groups to covalently bond to proteins and polysaccharides. These covalently bonded ascorbate forms would retain the enediol lactone ring and maintain its potential as a catalyst. The similarity in the ascorbate side chain and catecholamine metabolism with monoamine oxidase lead to many questions, including whether the catechols and ascorbate are degraded by similar enzymes.

Table I

Periodate Degradation Yield from 3.33 mmoles of Ascorbic Acid

	mmoles of Formaldehyde	mmoles of Oxalate	mmoles of Formate	mmoles of CO_2
Yield mM	3.12	3.36	5.15	3.30
Calculated yield mM	3.33	3.33	6.66	3.33
% Yield	93.5	100.8	77.0	99.0

Table II

Distribution of Radioactivity from Periodate Degradation of Ascorbic-6-^{14}C Acid, Ascorbic-1-^{14}C Acid, Dehydroascorbic-1-^{14}C Acid, and Ascorbate-6-^{14}C Sulfate

	Recovery	Formaldehyde	Oxalate	Formate	CO_2
1.2 μCi As-6-^{14}C	μCi	1.018	0.006	0.022	0.01
in 3.33 mmoles	Percent*	96.4	0.61	2.04	0.94
1.2 μCi As-6-^{14}C	μCi	1.104	0.006	0.002	0.002
in 2.5 mmoles	Percent*	99.1	0.53	0.21	0.20
2.5 μCi As-1-^{14}C	μCi	0.001	1.91	0.005	0.259
in 2.5 mmoles	Percent*	0.03	87.8	0.24	11.9
2.5 μCi As-1-^{14}C	μCi	0.001	1.13	0.003	0.238
in 2.5 mmoles	Percent*	0.04	82.4	0.21	17.4
2.5 μCi dAs-1-^{14}C	μCi	0.001	1.83	0.003	0.244
in 2.5 mmoles	Percent*	0.05	88.4	0.14	10.8
10. μCi As-6-^{14}C SO_4	μCi	6.79	0.037	0.34	0.026
in 2.5 mmoles	Percent*	94.4	0.50	4.7	0.4

*Calculated as percent to total carbon-14 recovered

Table III

Recovery of Formaldehyde-^{14}C from Periodate Degradation of Whole Rat Urine Containing Ascorbate-6-^{14}C Metabolites

Day	Total ^{14}C Prior to KIO_4	$H^{14}CHO$	% $H^{14}CHO$
2	114.46 x 10^3 µCi	85.28 x 10^3 µCi	74.5
9	9.62 x 10^3 µCi	6.00 x 10^3 µCi	62.4
18	2.30 x 10^3 µCi	1.27 x 10^3 µCi	55.1
23	1.28 x 10^3 µCi	0.71 x 10^3 µCi	55.6

Table IV

Distribution of Radioactivity from Periodate Degradation of DEAE-32 Column Chromatography Peaks of Urine from a Rat Given Ascorbic-6-^{14}C.

Peak	Total ^{14}C Prior to KIO_4	$^{14}CO_2$	$H^{14}CHO$	$^{-}OOC-^{14}COO^{-}$	$H^{14}COOH$	Carbon-14 Recovered
Neutral	2.96 x10^3µCi	0.04 x10^3µCi	2.04 x10^3µCi	0.36 x10^3µCi	0 µCi	2.44 x10^3µCi
Percent		1.4	68.9	12.2	0.0	82.4
Weak Acid	21.37 x10^3µCi	0 µCi	11.21 x10^3µCi	1.11 x10^3µCi	0 µCi	12.32 x10^3µCi
Percent		0.0	52.4	5.2	0.0	57.6
Peak II	23.48 x10^3µCi	0.03 x10^3µCi	3.25 x10^3µCi	0 µCi	0 µCi	3.28 x10^3µCi
Percent		0.1	13.9	0.0	0.0	14.0
Strip	8.65 x10^3µCi	0.01 x10^3µCi	1.70 x10^3µCi	6.13 x10^3µCi	0.81 x10^3µCi	8.65 x10^3µCi
Percent		0.1	19.6	70.9	9.4	100.

Table V

Distribution of Radioactivity from Periodate Degradation of DEAE-32 Column Chromatography Peaks of Day Two Urine from a Rat Given Ascorbic-6-^{14}C.

Peak	Total ^{14}C Prior to KIO_4	$^{14}CO_2$	$H^{14}CHO$	$^{-}OOC\text{-}^{14}COO^{-}$	$H^{14}COOH$	Carbon-14 Recovered
Neutral	15.55 x10^3µCi	0 µCi	10.70 x10^3µCi	0.81 x10^3µCi	0 µCi	11.51 x10^3µCi
Percent		0.0	68.8	5.2	0.0	74.0
Weak Acid	47.12 x10^3µCi	0 µCi	26.24 x10^3µCi	0.63 x10^3µCi	0 µCi	26.87 x10^3µCi
Percent		0.0	55.7	1.3	0.0	57.0
Peak I	7.40 x10^3µCi	0.03 x10^3µCi	7.37 x10^3µCi	0 µCi	0 µCi	7.40 x10^3µCi
Percent		0.4	99.6	0.0	0.0	100.
Peak II	7.85 x10^3µCi	0 µCi	5.26 x10^3µCi	0.54 x10^3µCi	0 µCi	5.80 x10^3µCi
Percent		0.0	71.1	7.3	0.0	78.4

Table VI

Recovery of Formaldehyde-^{14}C from Periodate Degradation of Whole Monkey Urine Containing Ascorbate-6-^{14}C Metabolites

Day	Total ^{14}C Prior to KIO_4	$H^{14}CHO$	% $H^{14}CHO$
1	82.51 x10^3µCi	45.22 x10^3µCi	54.8
6	17.21 x10^3µCi	8.45 x10^3µCi	49.1
29	16.70 x10^3µCi	9.76 x10^3µCi	58.4
39	9.20 x10^3µCi	5.24 x10^3µCi	57.0
41	7.24 x10^3µCi	3.70 x10^3µCi	51.1
43	15.85 x10^3µCi	8.76 x10^3µCi	55.3
57	7.89 x10^3µCi	3.76 x10^3µCi	47.6

Table VII

Distribution of Radioactivity from Periodate Degradation of DEAE-32 Column Chromatography Peaks of Day One Urine from a Macaque Monkey Given Ascorbic-6-^{14}C, i.v.

Peak	Total ^{14}C Prior to KIO_4	$^{14}CO_2$	$H^{14}CHO$	$^{-}OOC\text{-}^{14}COO^{-}$	$H^{14}COOH$	Carbon-14 Recovered
Neutral	3.29 x10^3µCi	0 µCi	1.56 x10^3µCi	0 µCi	0 µCi	1.56 x10^3µCi
Percent		0.0	47.5	0.0	0.0	47.5
Weak Acid	28.35 x10^3µCi	0.30 x10^3µCi	19.78 x10^3µCi	0 µCi	0 µCi	20.08 x10^3µCi
Percent		1.1	69.8	0.0	0.0	70.8
$AsSO_4$	2.07 x10^3µCi	0 µCi	0.88 x10^3µCi	0.63 x10^3µCi	0 µCi	1.51 x10^3µCi
Percent		0.0	42.7	30.4	0.0	72.9
Strip	1.33 x10^3µCi	0 µCi	1.27 x10^3µCi	0 µCi	0 µCi	1.27 x10^3µCi
Percent		0.0	95.8	0.0	0.0	95.8

Figure I. Urine ^{14}C Excretion for a Rat Given 33 µCi, 10 mg, Ascorbic-6-^{14}C Acid, subcutaneously.

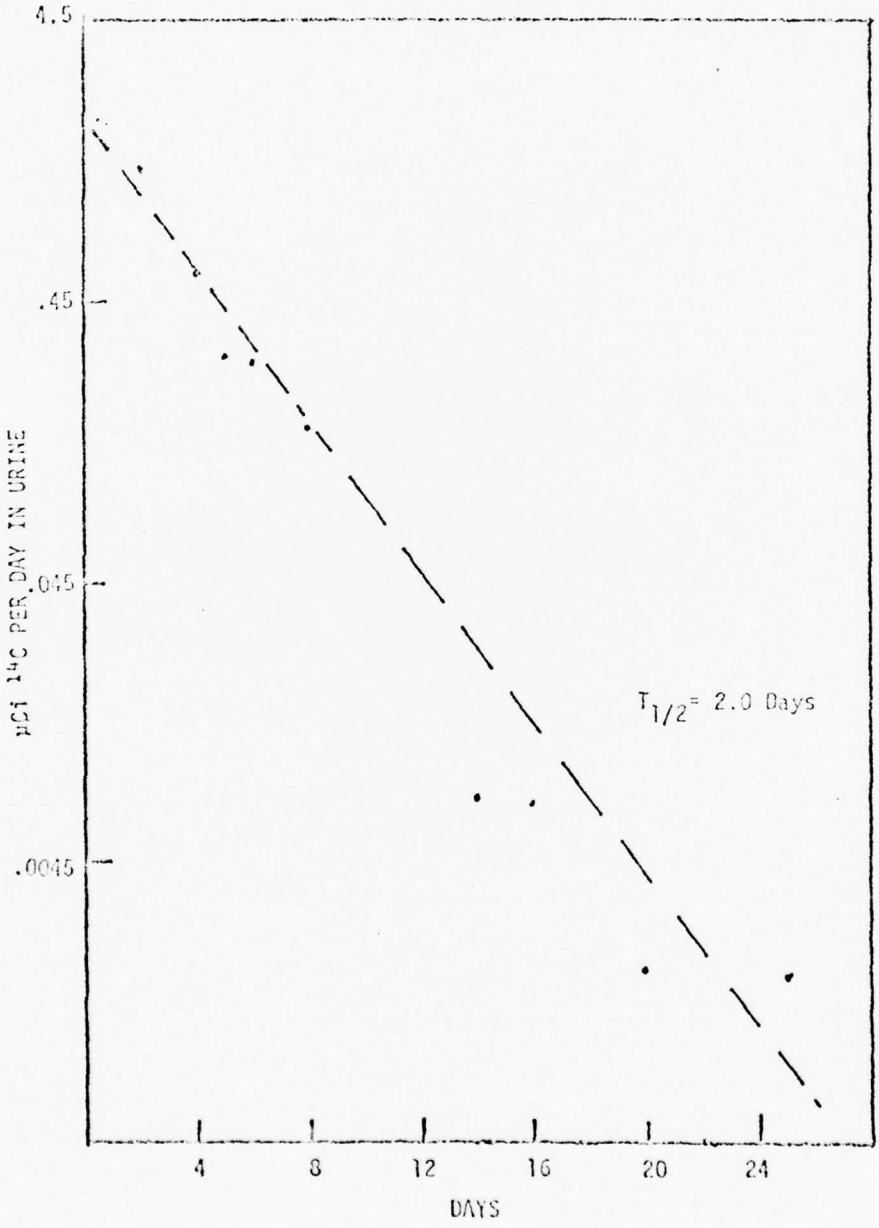

4.5
.45
.045
.0045
μCi 14C PER DAY IN URINE
$T_{1/2}$= 2.0 Days
4
8
12
16
20
24
DAYS

Figure II. DEAE-32 Column Chromatograph of Day One Urine from a Rat Given 33 μCi, 10 mg, Ascorbic-6-^{14}C Acid, subcutaneously.

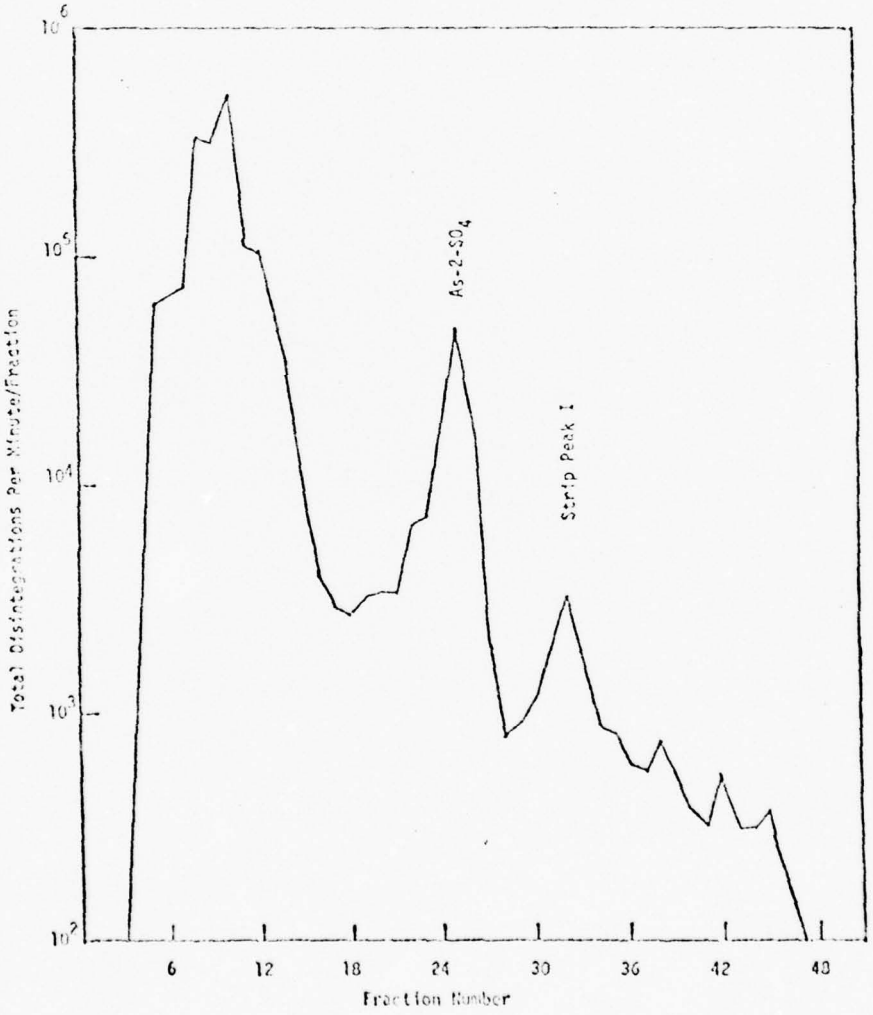

10^6
10^5
10^4
10^3
10^2
Total Disintegrations Per Minute/Fraction
As-2-SO$_4$
Strip Peak I
6
12
18
24
30
36
42
48
Fraction Number

Figure III. DEAE-32 Column Chromatograph of Day One Urine from a Rat Given 33 μCi, 10 mg, Ascorbic-6-^{14}C Acid, subcutaneously.

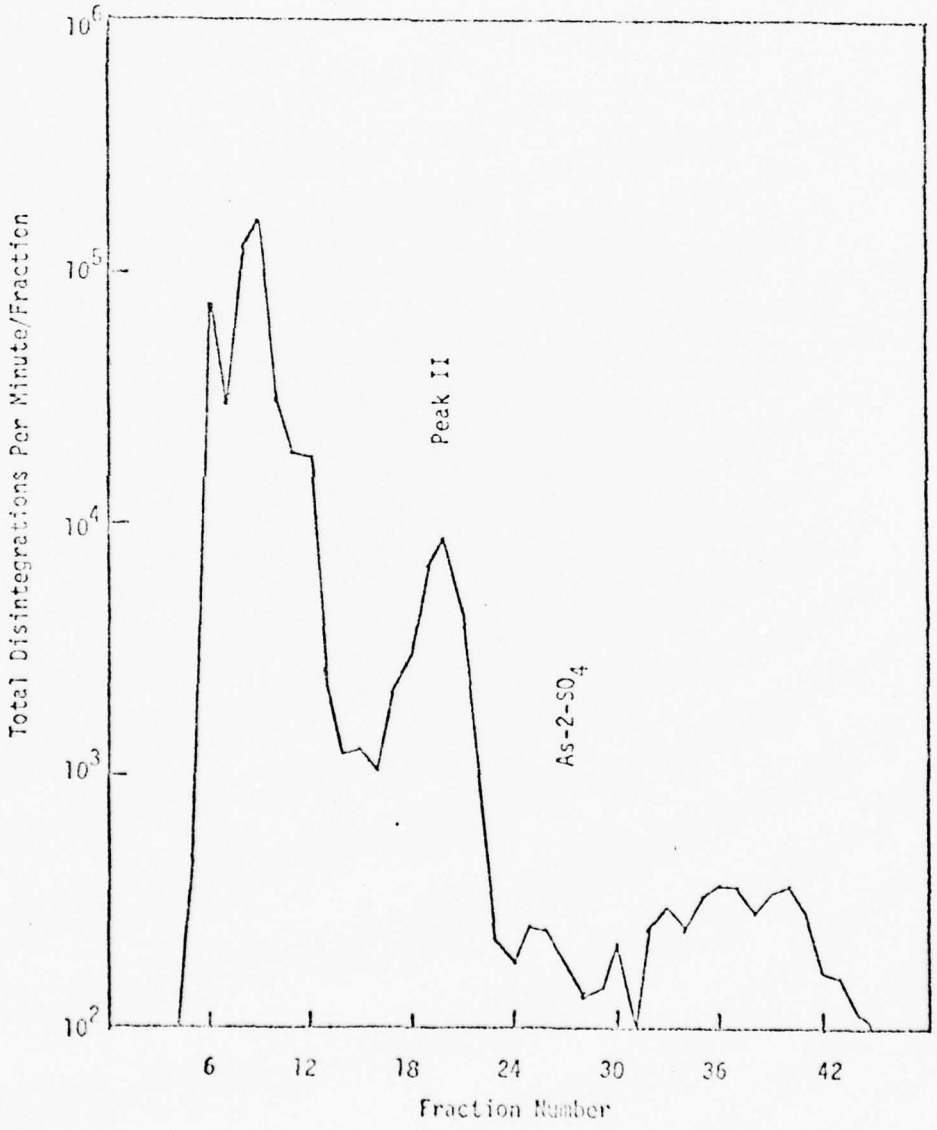

Total Disintegrations Per Minute/Fraction
10^6
10^5
10^4
10^3
10^2
Peak II
As-2-SO_4
6
12
18
24
30
36
42
Fraction Number

Figure IV. DEAE-32 Column Chromatograph of Day Three Urine from a Rat Given 33 μCi, 10 mg, Ascorbic-6-^{14}C Acid, subcutaneously.

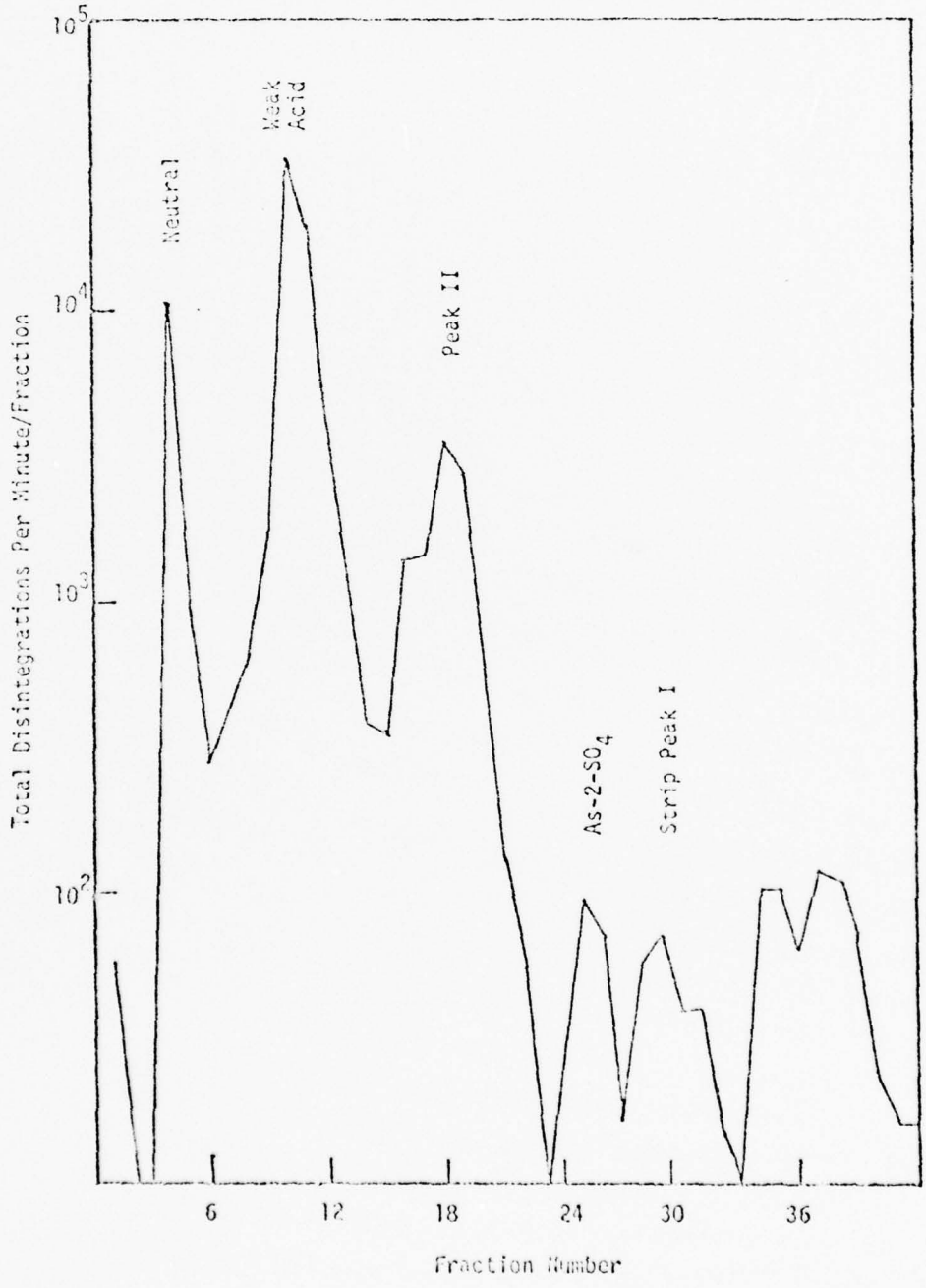

Total Disintegrations Per Minute/Fraction
10^5
10^4
10^3
10^2
Neutral
Weak Acid
Peak II
As-2-SO_4
Strip Peak I
6
12
18
24
30
36
Fraction Number

Figure V. DEAE-32 Column Chromatograph of Day Two Urine from a Rat Given 33 µCi, 10 mg. Ascorbic-6-^{14}C Acid, subcutaneously.

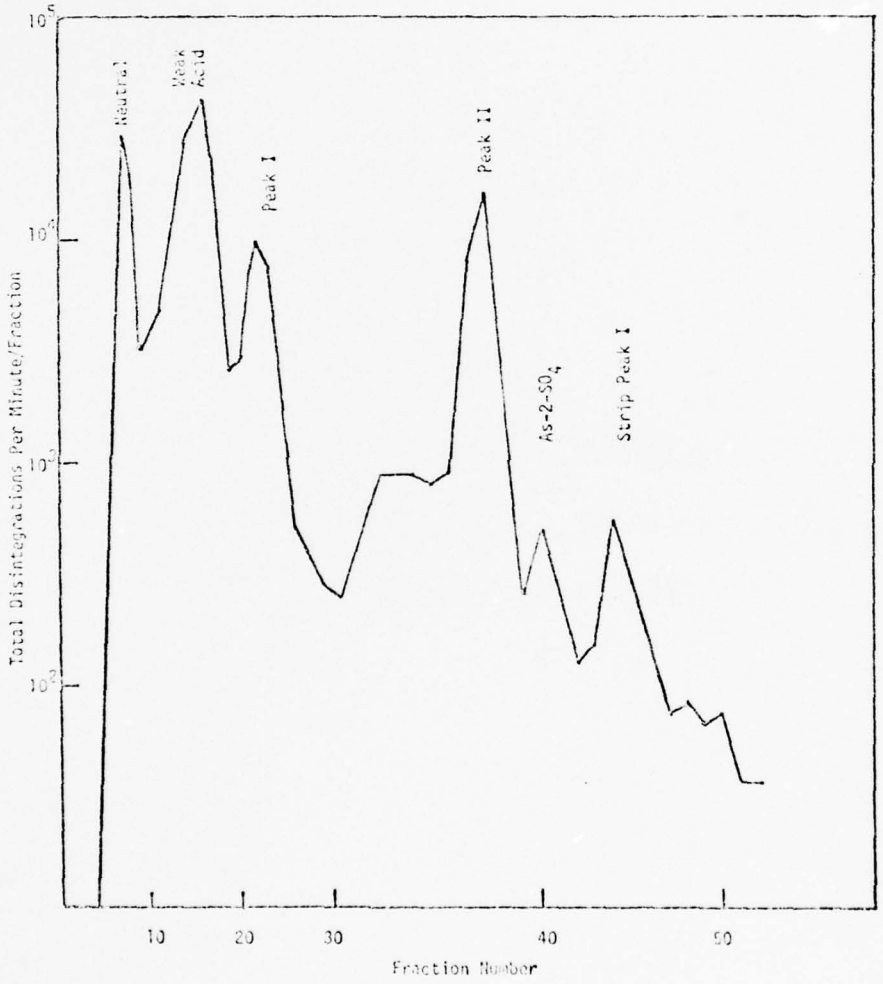

Neutral
Weak Acid
Peak I
Peak II
As-2-SO4
Strip Peak I
Total Disintegrations Per Minute/Fraction
Fraction Number
10
20
30
40
50

Figure VI. Urine ^{14}C Excretion for a Macaque Monkey Given 65 μCi, 25 mg, Ascorbic-6-^{14}C Acid, i.v.

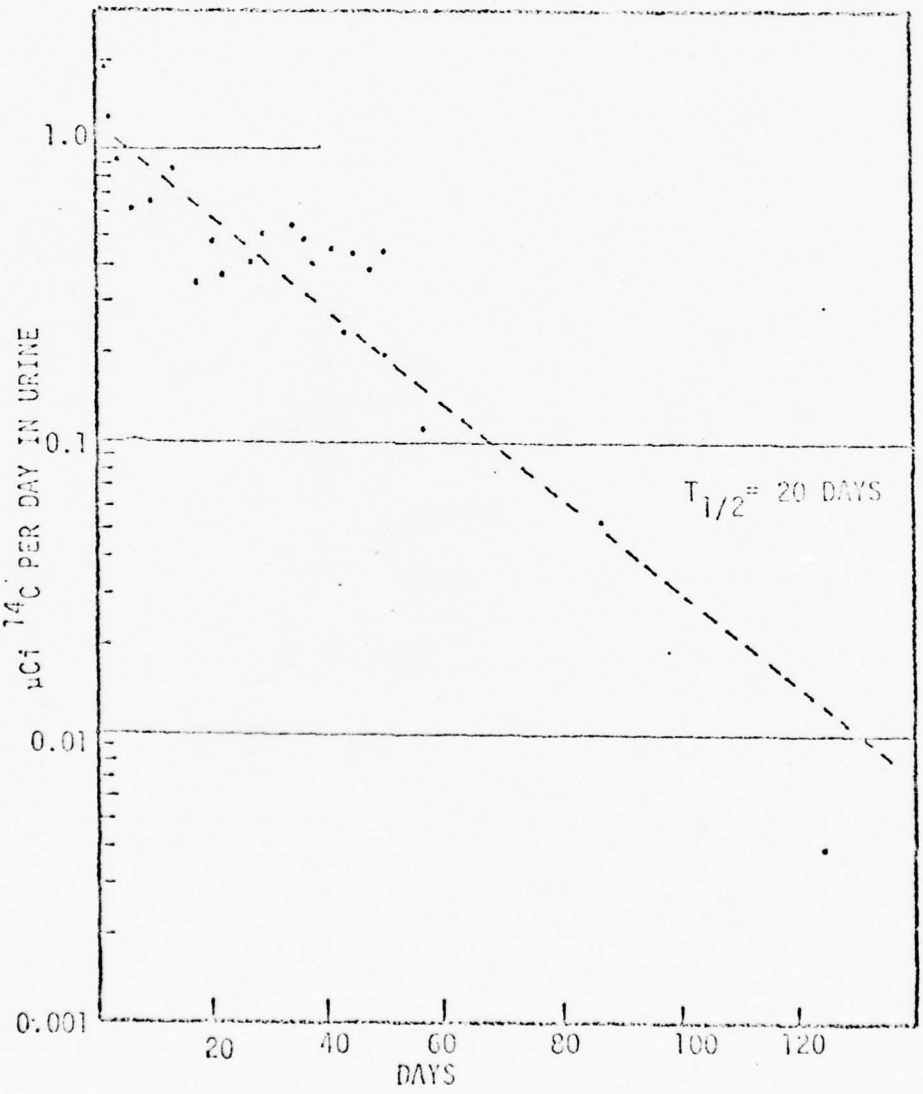
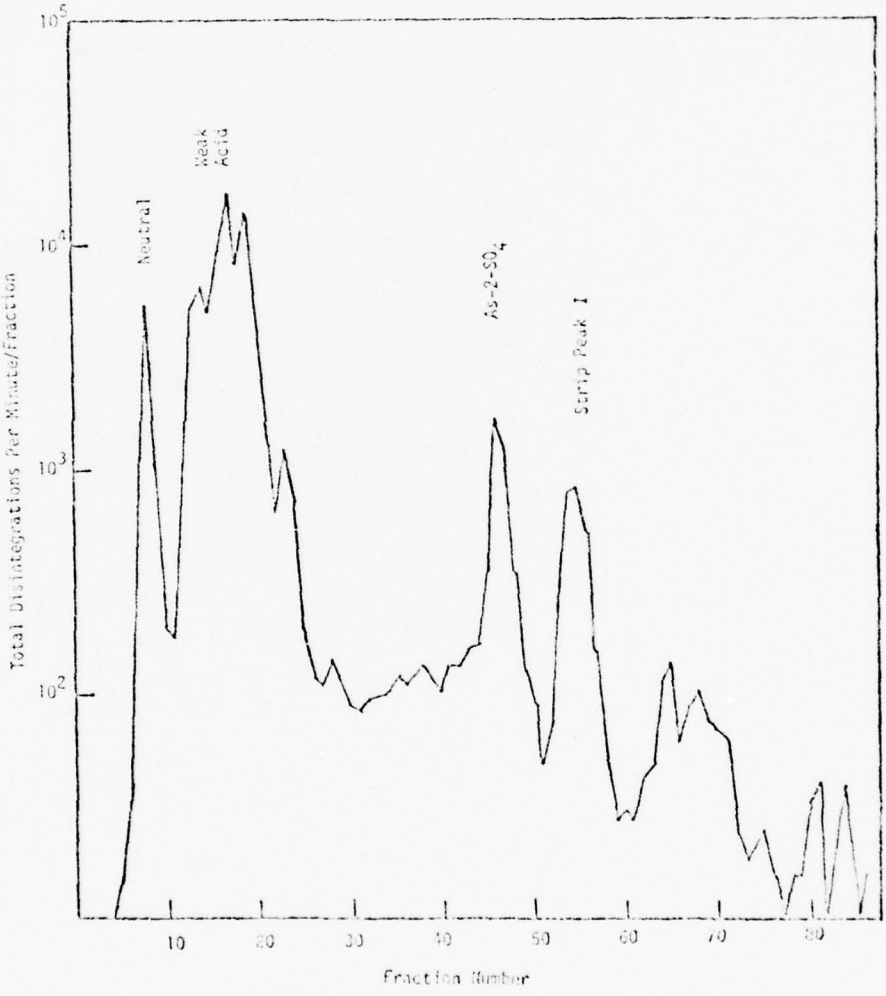

1.0
0.1
0.01
0.001
μCi ^{14}C PER DAY IN URINE
$T_{1/2}$= 20 DAYS
20
40
60
80
100
120
DAYS

Figure VII. DEAE-32 Column Chromatograph of Day One Urine from a Macaque Monkey Given 65 µCi, 25 mg, Ascorbic-6-^{14}C Acid, i.v.


Total Disintegrations Per Minute/Fraction
10^5
10^4
10^3
10^2
Neutral
Weak Acid
As-2-SO_4
Strip Peak I
10
20
30
40
50
60
70
80
Fraction Number

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Part V

Vol. 68, No. 3, 1976 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

ASCORBATE-2-PHOSPHATE INHIBITION OF ASCORBATE-2-SULFATE SULFOHYDROLASE FROM BOVINE LIVER

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Summary. The hydrolysis of ascorbate-2-sulfate by the enzyme, ascorbate-2-sulfate sulfohydrolase, purified from bovine liver has been shown to be powerfully inhibited by ascorbate-2-phosphate. The inhibition by ascorbate phosphate is competitive with a K_i of 0.3 uM. Na_2HPO_4 also inhibits by an apparent non-competitive process. The Na_2HPO_4 concentration at 50% inhibition is 7.7 uM. A possible control role for ascorbate phosphate in ascorbate biochemistry is suggested.

Ascorbate-2-sulfate is a naturally occurring metabolite of ascorbic acid which was first found in brine shrimp cysts and since then in the urine of man, monkeys, rats, and guinea pigs (1, 2, 3, 4, 5). It is probably a storage form of ascorbate in trout, and in fact relieves scurvy in trout (2).

The enzymic hydrolysis of ascorbate sulfate was first observed by Bullen using a crude arylsulfatase A preparation from bovine liver (6). Since then, ascorbate sulfate sulfohydrolase has been partially purified and characterized from bovine liver (7, 8) and from a marine gastropod (9, 10, 11). In both of these cases, the ascorbate sulfate sufohydrolase activity co-purified with arylsulfatase A activity measured using p-nitrocatechol sulfate as a substrate. Ascorbate sulfate is a good substrate for purified arylsulfatase A (12). Both ascorbate sulfate sulfohydrolase and arylsulfatase activities are inhibited by ascorbate phosphate and by Na_2HPO_4. However, arylsulfatase is inhibited to a lesser degree than ascorbate sulfate sulfohydrolase (unpublished data). In the case of the marine gastropod, ascorbate

sulfate sulfonydrolase is strongly inhibited by inorganic phosphate and by several nucleotides (11).

We report here a very powerful inhibition of a highly purified ascorbate sulfate sulfonydrolase from bovine liver by ascorbate phosphate and Na_2HPO_4. The inhibition by ascorbate phosphate is stronger than the inhibition by Na_2HPO_4. The possible role of ascorbate phosphate in ascorbate biochemistry is discussed.

MATERIALS AND METHODS

Ascorbate-2-sulfate and Ascorbate-2-phosphate. Ascorbate-2-sulfate and ascorbate-2-phosphate are prepared by direct sulfation or phosphorylation using $(CH_3)_3N{\cdot}SO_3$ (5) and $POCl_3$ respectively.

Ascorbate-2-sulfate Sulfohydrolase. The enzyme used in these studies has been purified 40,000 fold from bovine liver by procedures which are in preparation for publication. Arylsulfatase A activity co-purifies with ascorbate sulfate sulfonydrolase throughout these procedures.

Enzyme Assay. The enzyme assay consists of following the reduction of 2,6-dichloroindophenol by the ascorbate which is produced during the enzymic hydrolysis. The decrease in absorbance is followed at 516 nm which is the isobestic point of 2,6-dichloroindophenol. The pH of the assay reaction is pH 4.8 which is also the optimum. Since ascorbate sulfate is slowly acid hydrolyzed at this pH, a non-enzymic hydrolysis is used as a control. In the kinetic studies, 1.5 ml of 0.15 mM 2,6-dichloroindophenol in 0.05 M KAc pH 4.8 are placed in a 3 ml cuvette. To this is added an appropriate amount of 70 mM dipotassium ascorbate sulfate, 0.5 M NaAc or KAc, and water to give a total volume of 2 ml and an ionic strength of 0.1. Trisodium ascorbate phosphate and Na_2HPO_4 are added so that their final concentrations are from 0.2 to 0.4 uM and from 7.7 to 14.8 uM respectively. To the cuvette is added 0.05 ml of a 40/1 dilution of purified enzyme solution which had an initial protein concentration of 0.26 mg/ml (determined by the Lowry method using BSA as a standard). For each substrate concentration the non-enzymic hydrolysis rate is subtracted from the hydrolysis rate with the enzyme to give the true rate of enzymic hydrolysis. The rate of reaction at each substrate concentration is measured three to four times.

RESULTS AND DISCUSSION

Figure 1 (Top) shows that the Km for ascorbate sulfate is 12 mM, and that the inhibition by ascorbate phosphate is competitive and very powerful with a K_I of 0.3 uM. Figure 1 (Bottom) shows the inhibition by Na_2HPO_4. The intersection point is to the left of the 1/v axis which indicates non-competitive inhibition. However, the area of intersection is close to the zero line and a small systematic error could give competitive inhibition. Thus the type of inhibition by Na_2HPO_4 is questionable. The reaction is inhibited 50% by 7.7 uM Na_2HPO_4.

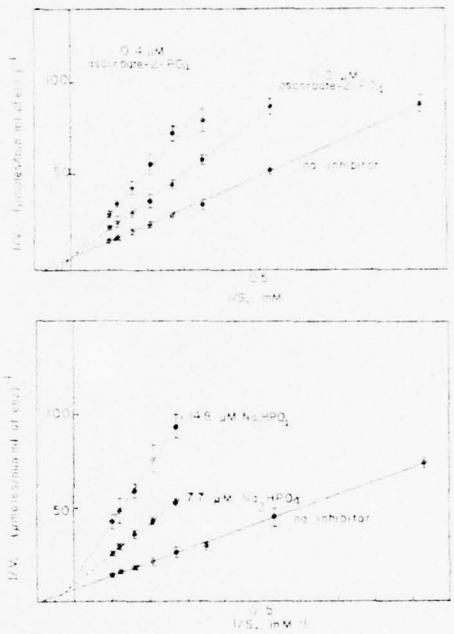

Figure 1. Top: A Lineweaver-Burke plot showing the inhibition of ascorbate-2-sulfate sulfohydrolase by ascorbate-2-phosphate. Bottom: A Lineweaver-Burke plot showing the inhibition of ascorbate-2-sulfate sulfohydrolase by Na_2HPO_4. The dashed lines are with the indicated inhibitor concentration and the solid lines are without inhibitor.

These results are summarized on Table I. The inhibition by ascorbate phosphate is approximately 25 fold more powerful than the inhibition by Na_2HPO_4. The K_I for ascorbate phosphate is 40,000 fold lower than the Km. The powerful inhibition of ascorbate sulfate sulfohydrolase by ascorbate phosphate with a K_I of 0.3 μM shows that ascorbate phosphate is bound very tightly to the enzyme. The fact that this binding is 25 fold more powerful than it is for Na_2HPO_4 and that the type of inhibition by ascorbate phosphate is different from Na_2HPO_4 suggests that the binding of ascorbate phosphate is quite specific.

Ascorbate phosphate is an interesting molecule. It is stable for weeks in neutral aqueous solution. It can be used to cure scurvy in the guinea pig (13). It is subject to rapid hydrolysis by alkaline phosphatase (unpub-

TABLE I

Inhibition of Ascorbate-2-Sulfate Sulfohydrolase by Na_2HPO_4 and by Ascorbate-2-Phosphate.

Inhibitor	K_I or Inhibitor Concentration at 50% Inhibition $[I]_{50\%}$	Type of Inhibition
None	Km = 12 mM	---
Ascorbate-2-phosphate	K_I = 0.3 μM	Competitive
Na_2HPO_4	$[I]_{50\%}$ = 7.7 μM	Non-competitive[a]

[a]The type of inhibition by Na_2HPO_4 is questionable. This is explained in the text.

lished data) and probably many other phosphatases since it is unstable in biological fluids. Ascorbate phosphate should be a moderately high energy compound, yet it would have a short life time in biological tissue. These properties, coupled with the very tight binding of ascorbate phosphate reported above, lead us to suggest that ascorbate phosphate may function as an active form of ascorbic acid under biological conditions.

Ascorbate phosphate could serve as a control compound for the ascorbate sulfatase function described in this report. An example of such a control might be present in the trout. Ascorbate sulfate prevents scurvy in trout and salmon (2) by a process in which ascorbate is derived from ascorbate sulfate. Ascorbate phosphate is a likely candidate for control of the regeneration if the enzyme is similar to that described in this paper. The enzyme described in this paper, ascorbate sulfate sulfohydrolase, seems to be part of the arylsulfatase A enzymes (EC 3.1.6.1) and its major biological roles are uncertain. Details of the properties of this enzyme will be published later.

ACKNOWLEDGMENT

This work is supported in part by a contract with the Surgeon General's Office of the U. S. Army.

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C-6 OXIDATION OF ASCORBIC ACID: A MAJOR METABOLIC PROCESS IN ANIMALS

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Summary. Metabolic activity of the C-6 carbon of ascorbic acid has been examined using two experimental methods. 43% of the label from ascorbic-6-^{3}H acid is converted to water in the monkey after i.v. injection. The rate of formation of $^{3}H_2O$ corresponds to the rate of excretion of organic ascorbate metabolites in the urine as measured using ascorbic-6-^{3}H or ascorbic-6-^{14}C acid. Periodate degradations of whole urine from rats or monkeys given ascorbic-6-^{14}C acid by i.v. injection show that 45% of the labeled metabolites do not give formaldehyde from the C-6 carbon, indicating the C-6 carbon is no longer a primary alcohol or that the C-5 and/or C-6 hydroxyl groups are derivitized. There is no significant excretion of $^{14}CO_2$ in these experiments indicating the metabolites do not enter general carbohydrate catabolism. The combination of these results show that about 45% of the C-6 carbon of ascorbic acid is oxidized *in vivo*. Because the monkeys had an adequate dietary intake of ascorbic acid and the rats synthesize ascorbic acid as needed, the results also indicate that the precursor of the oxidation is a slowly exchanging form of ascorbic acid in which the C-6 carbon is in the primary alcohol oxidation state.

Metabolic activity of the side chain of ascorbic acid, carbons 5 and 6, has not normally been considered in ascorbic acid biochemistry in higher animals. Several studies indicate such a process should be further explored. Hornig (1) suggests that the 6-carboxy derivative of ascorbate-2-sulfate might be a metabolite of ascorbic acid. Loewus (2) has suggested oxidation-reduction of the C-5 carbon of ascorbate precursors in some plants. We find the number and diversity of urinary metabolites of ascorbic acid in higher animals is difficult to explain without suggesting C-6 oxidized metabolites (3), (4).

In this paper we describe results from two experimental approaches which provide clear evidence that the side chain of ascorbic acid is metabolically active and subject to C-6 oxidation. Biological implications of these observations are discussed.

MATERIALS AND METHODS

Labeled Compounds. Ascorbic-6-^{14}C acid was made as described (5). Ascorbic-6-^{3}H acid was a gift from Drs. U. Gloor and F. Weber, F. Hoffman-LaRoche & Co., Basle, Switzerland.

Animal Procedures. Macaque monkeys weighing about 3 kg were injected i.v. with labeled ascorbic acid after 12 hours on no food. No food was given for the next 24 hours. They were then fed a normal monkey ration ad lib which provided about 300 mg ascorbic acid per day. 24 hour urines were collected at intervals. Where appropriate, breath $^{14}CO_2$ measurements were made at intervals. Rats were injected subcutaneously with labeled ascorbic acid and 24 hour urines collected. Injected amounts were: monkeys, 253 uCi in 50 mg ascorbic-6-^{3}H; monkeys, 62.3 uCi in 25 mg ascorbic-6-^{14}C; rats, 33.2 uCi in 10 mg ascorbic-6-^{14}C.

Assay of ^{3}H Labeled Urine. Urines were assayed for total radioactivity and for specific activity of water. From these values and urine volumes, 24 hour excretions of tritium in organic forms were calculated.

Assay of ^{14}C Labeled Urines. Total radioactivity was measured. Five to eight ml aliquots were filtered using a UM-2 filter, diluted to 25 ml, and 2.5 mM ascorbic acid added as carrier. Periodate degredation was done as described (6). Formaldehyde was isolated as the dimedone derivative and counted.

RESULTS AND DISCUSSION

Figure 1 shows the urine $^{3}H_2O$ specific activity and organic radioactivity for a monkey given ascorbic-6-^{3}H acid. The curves are parallel and follow a first order process with $T_{1/2}$ of 20 days. A short burst of organic-^{3}H in the initial days of the experiment is believed due to labeled impurities in the sample. In a duplicate experiment with another monkey a $T_{1/2}$ of 17 days was observed. The half time for excretion of ascorbic acid and metabolites has previously been reported as 20 d in both monkey (7) and man (8). On the average 27% of the daily ^{3}H excretion in urine is present as H_2O. In addition the monkey excreted $^{3}H_2O$ as insensible loss from breath and skin. Experiments using

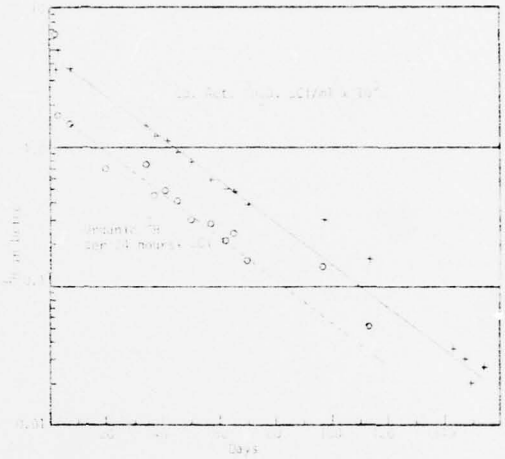


Figure 1. Urinary excretion of ^{3}H after i.v. injection of ascorbic-6-^{3}H acid in a macaque monkey: solid line with crosses, specific activity of urine water, uCi/ml x 10^{3}; dashed line with circles, total uCi organic ^{3}H excreted per 24 hours. The organic tritium values after 120 days were too low to measure reliably. $T_{1/2}$ for these excretion processes is 20 days.

alcohol-Dry Ice-cooled traps to collect such water show that these losses are about equal to urine $^{3}H_2O$. Thus the amount of ^{3}H excreted as water is 40% of the total. These data clearly show that the C-6 carbon of ascorbic acid is metabolically active in a process that causes a significant release of the C-6 hydrogens.

Results of periodate degradation of urine from a rat and the monkey given ascorbic-6-^{14}C acid are shown in Table I. In both animals the late urines show that 45% of the radioactivity cannot be recovered as the formaldehyde derivative. Processes that could account for non-production of HCHO-^{14}C include a) oxidation of the C-6 carbon, b) derivitization of the 5 or 6 hydroxyl groups or c) reduction of the C-5 or C-6 carbons. Loss of ^{3}H

TABLE I

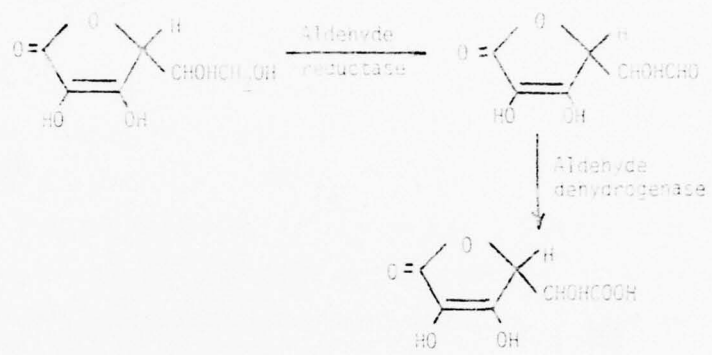
Periodate degradation of aliquots of 24 hour urine samples from a rat and a monkey given ascorbate-6-^{14}C. Percent of total ^{14}C that was recovered as the dimedone derivative of HCHO-^{14}C.

Monkey		Rat	
Day	HCHO-^{14}C	Day	HCHO-^{14}C
1	55%	2	75%
6	49	9	62
29	56	18	55
39	57	23	56
41	51		
43	55		
57	48		

to H_2O in the tritium experiments clearly show that the C-6 carbon is being oxidized.

The slow increase in percent C-6 carbon oxidized in the rat data indicates that the precursor of the C-6 oxidation process is a compound with a long turnover time. Such an inference also can be made from the monkey data. When the "ascorbate pool" of monkeys is labeled the half-time for excretion of the label is about 20 days, whether or not dietary ascorbic acid is given. Thus the labeled ascorbate pool does not exchange rapidly with dietary ascorbic acid. The parallel curves for ^3H_2O and organic ^3H in figure 1 indicate that the ^3H_2O must be released as the "ascorbate pool" is turned over. If the body water turnover time was similar to the ascorbate pool turnover time, this argument would fail, but other experiments by the authors show the body water turnover has a $T_{1/2}$ of about seven days in monkeys, distinctly different from the 20 d $T_{1/2}$ for ascorbate.

It seems reasonable to suggest the following metabolic process based on the above observations.



It is not known if the substrate for such a reaction is ascorbic acid itself or a derivative. The 2-methyl and 2-sulfate derivative are both known metabolites of ascorbate (9, 10).

This process is of biological interest for at least two reasons. First, C-6 oxidized derivatives of ascorbate have functional groups that allow easy covalent bonding of these metabolites to proteins and polysaccharides. Such covalently bonded forms of ascorbic acid would retain the unique enediol lactone ring in an unsubstituted form and potential catalytic functions of ascorbate itself.

Secondly, ascorbic acid and its postulated side chain oxidation products are structurally very similar to those observed in catecholamine catabolism. When norepinephrine is degraded by monoamine oxidase, the aldehyde formed is oxidized and reduced to an acid and alcohol. The alcohol, aldehyde and acid have the same side chain as our postulated ascorbate metabolites. In addition, the catechol ring and ascorbate ring have many properties in common, being highly conjugated, nearly planar, similar in size, and good reducing agents. Catechol-O-methyl transferase methylates both norepinephrine and ascorbate (9) and sulfated derivatives of both are observed. These structural

similarities lead to many questions, especially whether the catechols and ascorbate are degraded by similar enzymes and whether any intermediates are competitive or interactive.

ACKNOWLEDGEMENTS

The authors thank Dr. B. L. Horecker for suggesting the periodate degradation approach to this problem. We also thank Drs. U. Gloor and F. Weber for the ascorbic-6-^{2}H acid and Bennett M. Creeden, Robert Schlein and Anne G. Tolbert for technical assistance in these experiments. Supported in part by a contract with the U.S. Army Medical Research and Development Command.

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Part VII

The Presence of L-Threo-Saccharoascorbic Acid in Monkey Urine as a Metabolite of Ascorbic Acid

By William K. Sietsema

1976

Table of Contents

The purpose of the work discussed in this paper is to show the presence of L-threo-saccharoascorbic acid (SAs) (fig. 2) in the urine of monkeys that had been injected with labeled ascorbic acid (As) (fig. 1).

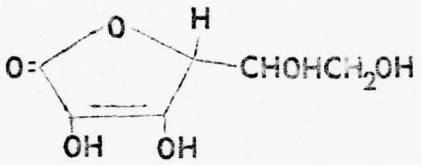


Figure 1. ascorbic acid

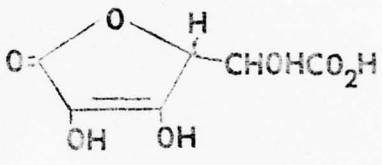


Figure 2. L-threo-saccharoascorbic acid

The data collected can be divided into two categories; that collected from thin layer chromatography plates, and that from column chromatography. One may note that the data from the TLC plates is in some ways inconclusive, but the column data shows some nice correlations, and may well serve as a valuable separation technique for ascorbic acid chemists.

The animal portion of these experiments was done for us by the Letterman Army Institute of Research in the Presidio, San Francisco. A 3 kg. macaque monkey, fed a normal diet (ad lib) with 300 mg. As/day was given 20 mg. of As-6-^{14}C (65 μc.) intravenously. The urine

was collected daily and kept frozen until it was shipped to us packed in dry ice. It was stored here in a deep freeze.

PART I: THIN LAYER CHROMATOGRAPHY

Six TLC systems were developed by trial and error that would separate SAs from As. These systems were used to separate the labeled urine, along with standards. Autoradiographs were then made of the plates by scraping sections of silica gel and counting them with a liquid scintillation counter. Graphs made of the radioactivity versus fraction number revealed any possible correlations.

Methods and Materials

Making Silica Gel Plates: TLC plates were made using a spreader from "Quickfit Instruments" in England. Silica gel type H from Sigma Chemical Company was mixed with water in a ratio of 1:2 by weight. The mixture was then poured into the slide, and spread to a thickness of 1 mm. Gently dropping the entire spreader helped the bubbles to settle out. The plates were air dried at room temperature, and then stored in an oven at ~80°C. For more on thin layer chromatography, see Randerath (1).

Preparation of the Urine (2): Five ml. of the day 8 urine was filtered through a Diaflo membrane using

45 p.s.i. of nitrogen as a propellant. This urine was blown down to 1 ml. at 37°C under more N_2. Next, it was run through a DEAE-32 column in the sulfate form (30 X ¾ cm.) with the following gradients:

1) 75 ml. each 0.006 M H_2SO_4 and 0.009 M H_2SO_4
2) 50 ml. each 0.009 M H_2SO_4 and stripper
3) 30 ml. stripper (0.2 M Na_2SO_4 in 0.002 M H_2SO_4)

Fractions were collected, counted for radioactivity, and frozen. A graph was made of the radioactivity (DPM) versus fraction number (see Figure 3).

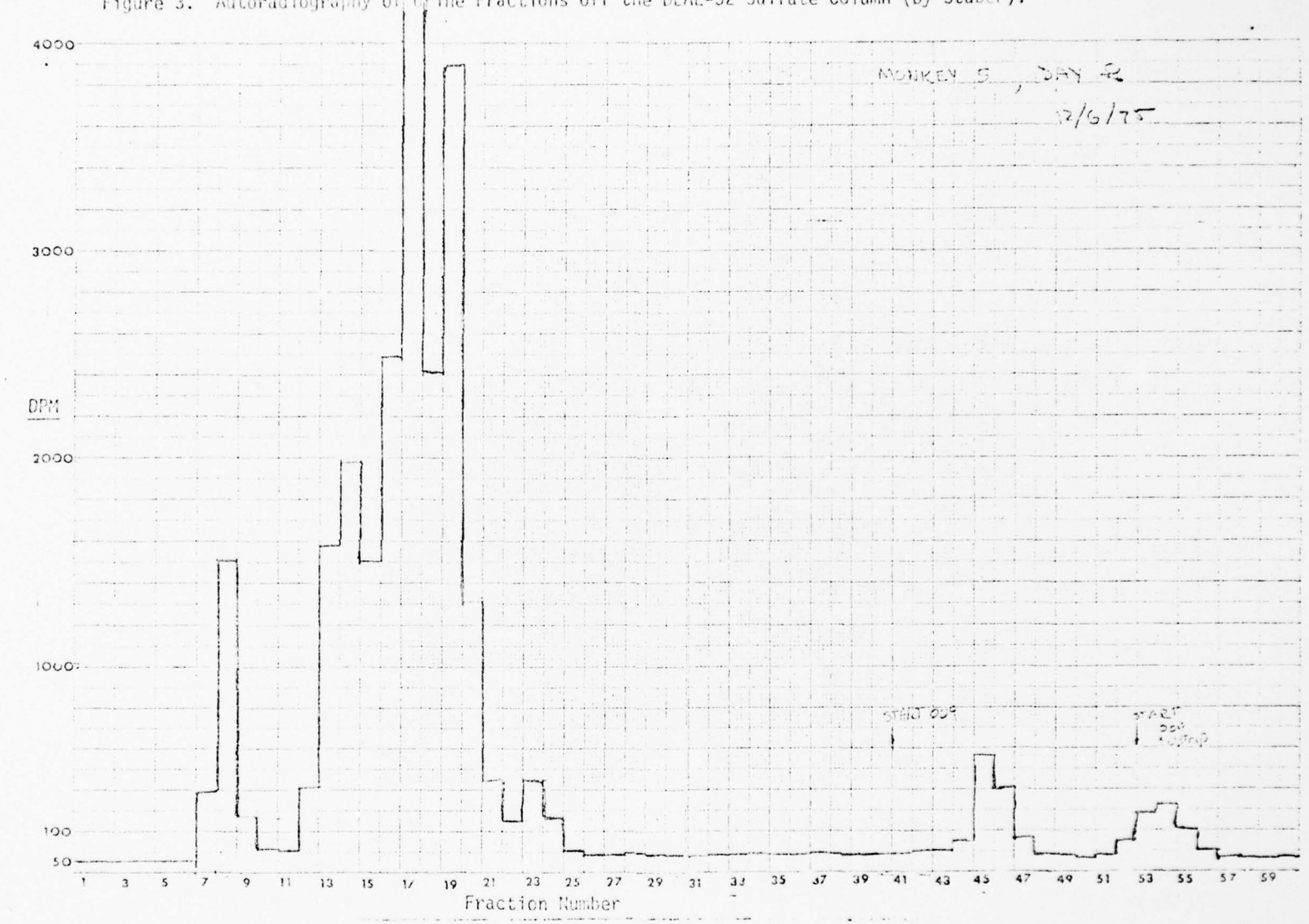
In order to simplify the TLC plate data, the fractions were divided into groups, using each major peak as 1 group (see Table 1). Each group was thawed, pooled, and rotovaped to a small volume. N_2 was used to further reduce the volume until a precipitate began forming. Water was added to just clear the solution.

Table 1. Division of radioactive peaks into groups.

Group	Fraction numbers
1	6-10
2	11-15
3	16-18
4	19-25

Spotting and Elution of TLC Plates: TLC plates were prepared by drawing vertical lines with a razor blade

Figure 3. Autoradiography of Urine Fractions off the DEAE-32 Sulfate Column (by Stuber).



1 cm. apart across the entire plate. Spotting was done between the vertical lines, 1.5 cm. from the bottom. The sample was spotted in the center, and standards were spotted on either side of this. The plate was placed in 200 ml. of freshly mixed solvent (see Table 2) and allowed to elute within 2 cm. of the top (2-5 hours).

Table 2. Solvent Systems

System	Ratio	Abbreviation
*Chloroform:Methanol:Water:Acetic Acid	65:50:15:2	CMWA
Acetic Acid:Ethanol:Benzene	10:50:40	AEB
*Acetone:Methanol:Benzene:Acetic Acid	5:20:20:5	AMBA
*n-Butanol:Acetic Acid:Carbon Tetrachloride	75:15:15	BAT
Acetic Acid:Methanol:Benzene	10:50:40	AMB
n-Butanol:Acetic Acid:Chloroform	70:20:15	BAC
Acetone:Methanol:Benzene	10:50:40	AcMB

*Best Systems

The plate was removed, and the solvent front marked. In order to visualize the standards, but not the sample, the plate was sprayed with a 2% iodine:ethanol solution while covering the sample with a sheet of notebook paper. Data was recorded, after dividing the plate into zones for counting (see Figure 4).

Liquid Scintillation Counting: After the plates were divided into zones, each sample zone was scraped off

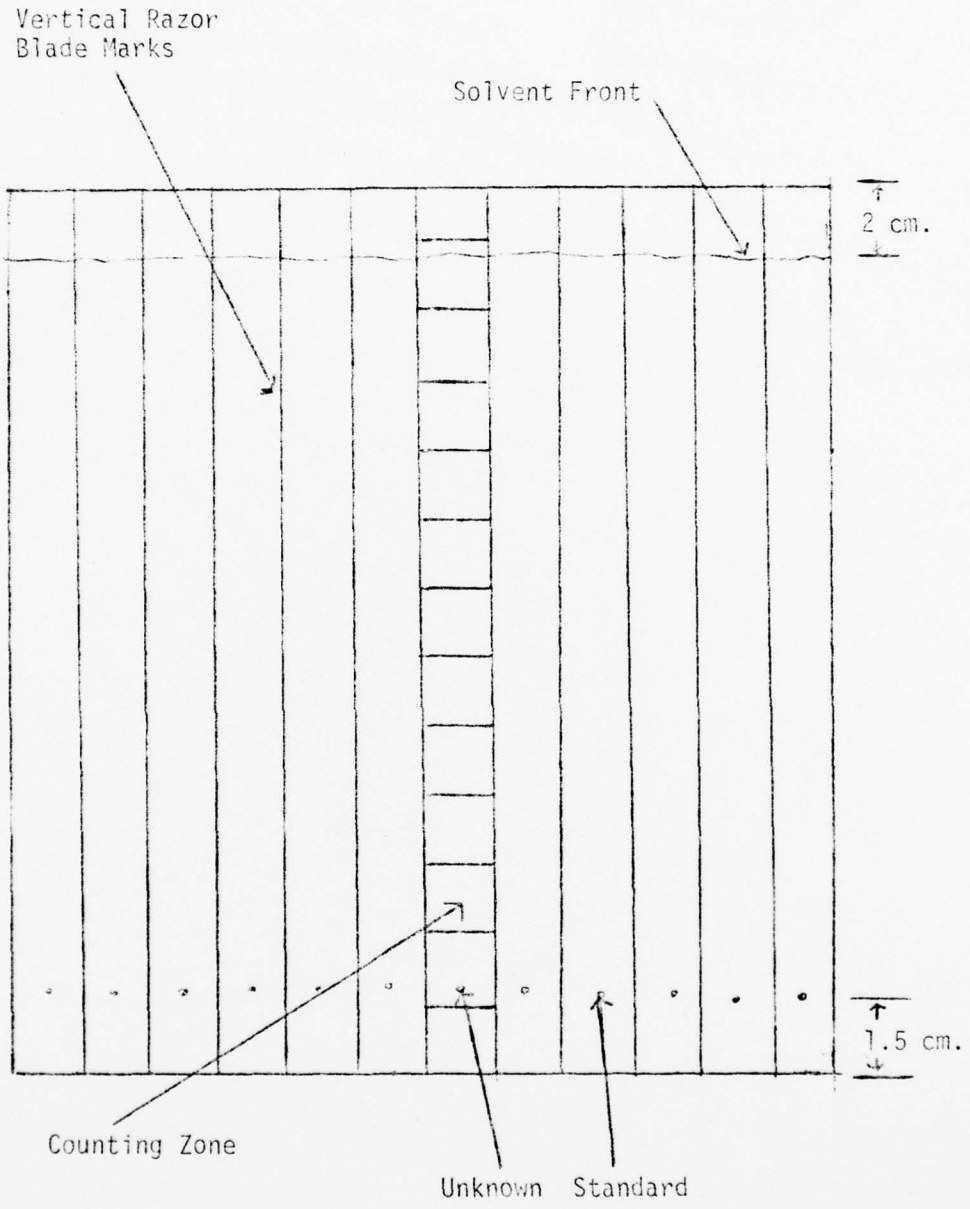


Figure 4. A typical TLC plate.

the plate into a glass counting vial, and mixed with 15 ml. of a counting solution. Two solutions were used, the first consisting of 200 gms. napthalene, 10 gms. 2,5-diphenyl-oxazole (PPO), and scintillation grade dioxane to make 2 liters. The second solution contained 8 gms. PPO, 150 mg. dimethyl POPOP, and scintillation grade toluene to make 2 liters. The dioxane solution had better solvation properties, but is more expensive to use, and is carcinogenic. Efficiency tests were used to correct the data, so the solution used is not too important.

Data

For simplicity, all the data is presented here in the form of graphs. The plots are of radioactivity versus fraction # (zone #). The position of standards is shown by horizontal lines drawn over the zones in which they were found. A peak located over a standard line is a correlation. It is suggested that one remove (carefully) the graphs, and place them on a large table so they can be rearranged when interpreting this data. The solvent system used is marked in the upper left hand corner of each graph, using the abbreviations found in Table 2. The group number is found underneath the solvent system.

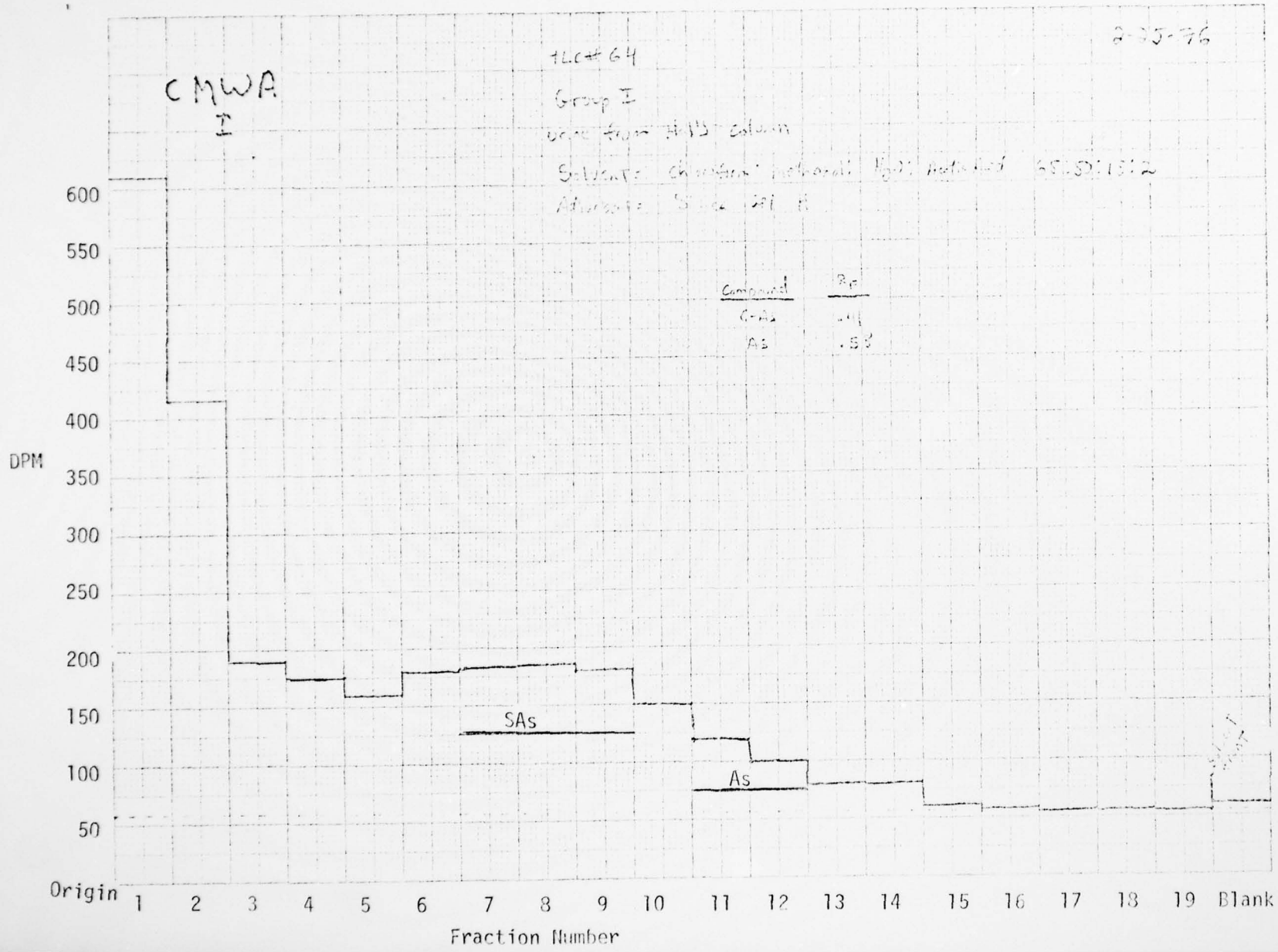

CMWA
I
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Figure 5.

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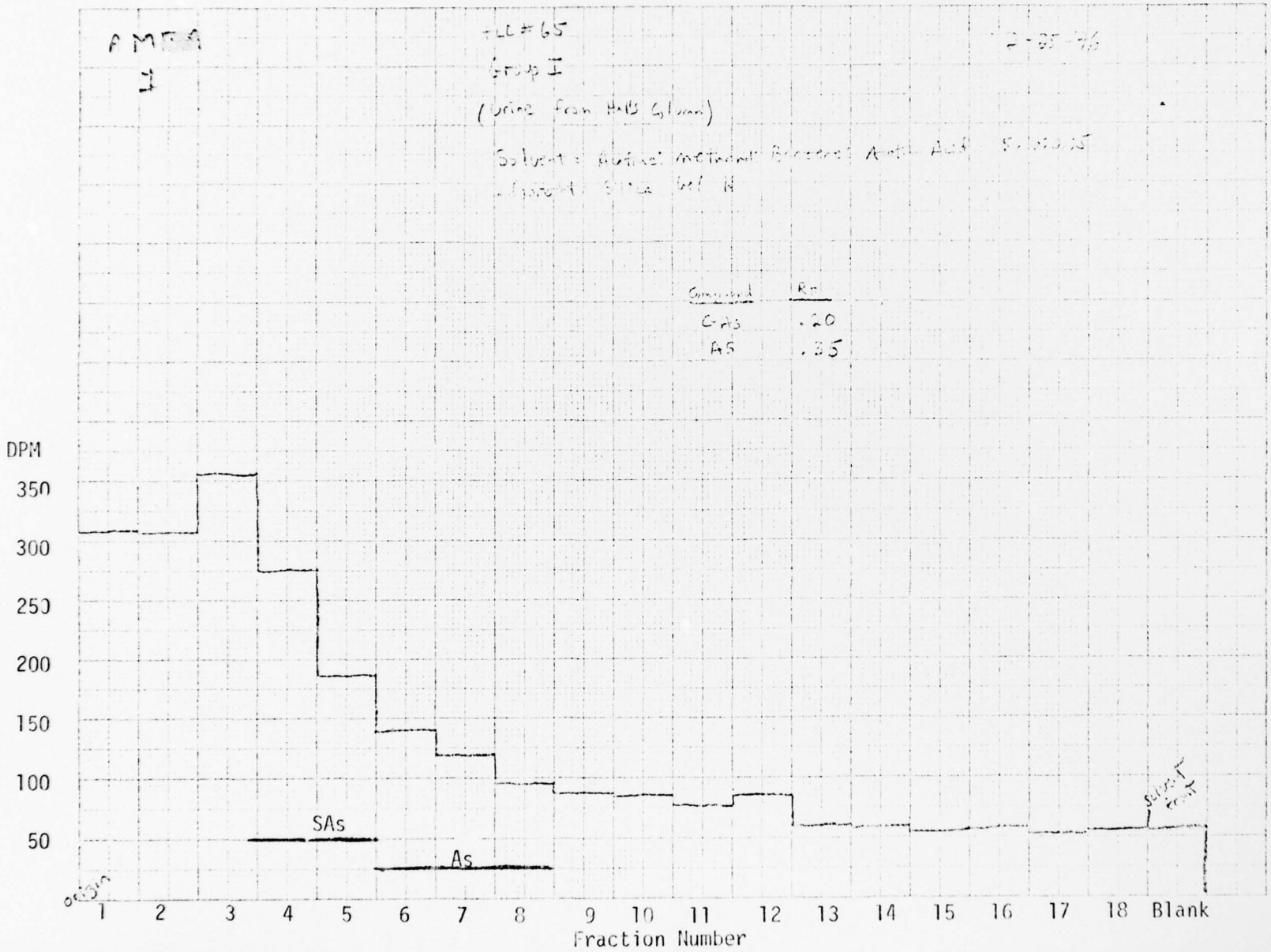
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Figure 6.

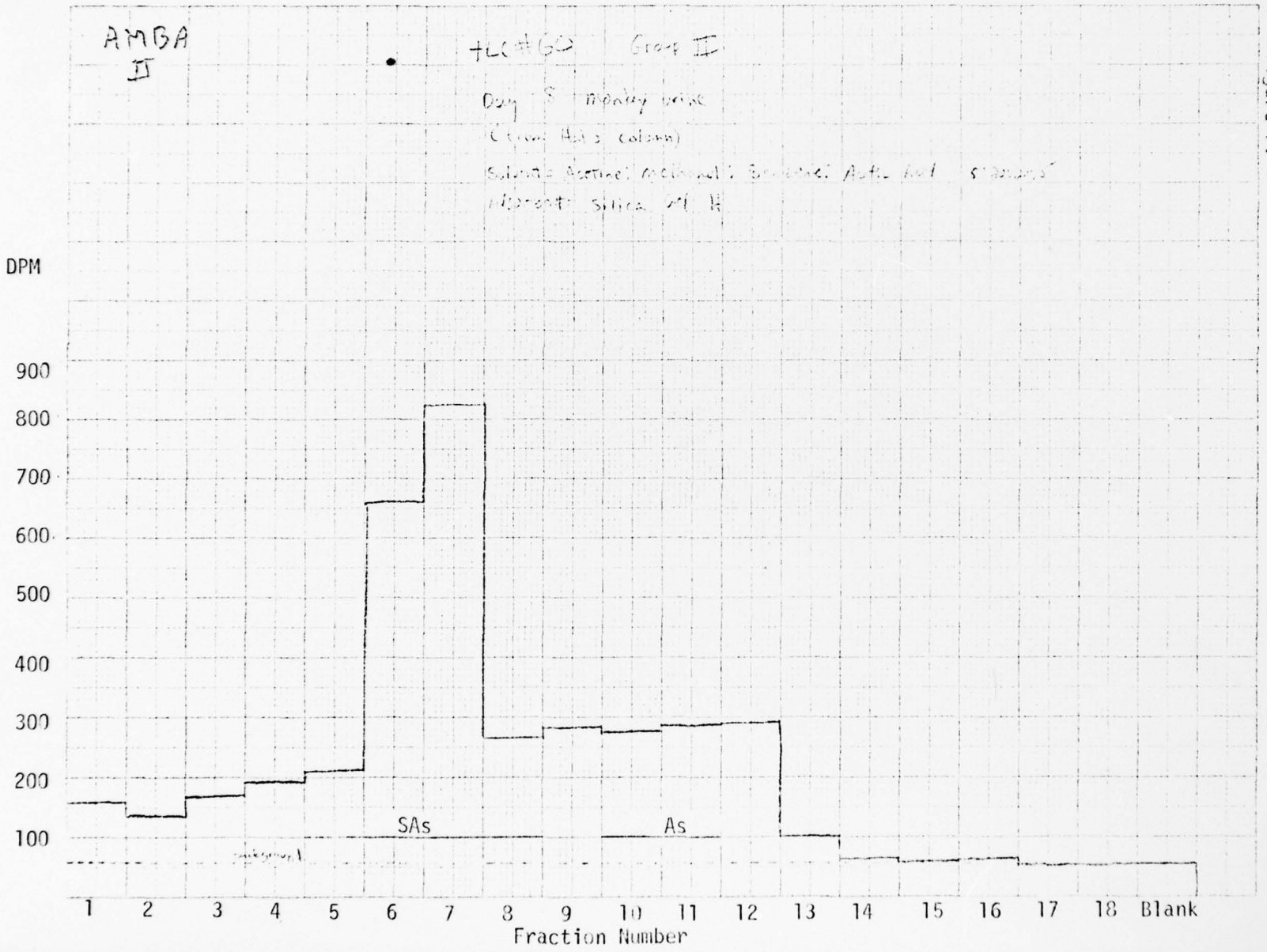
AMBA
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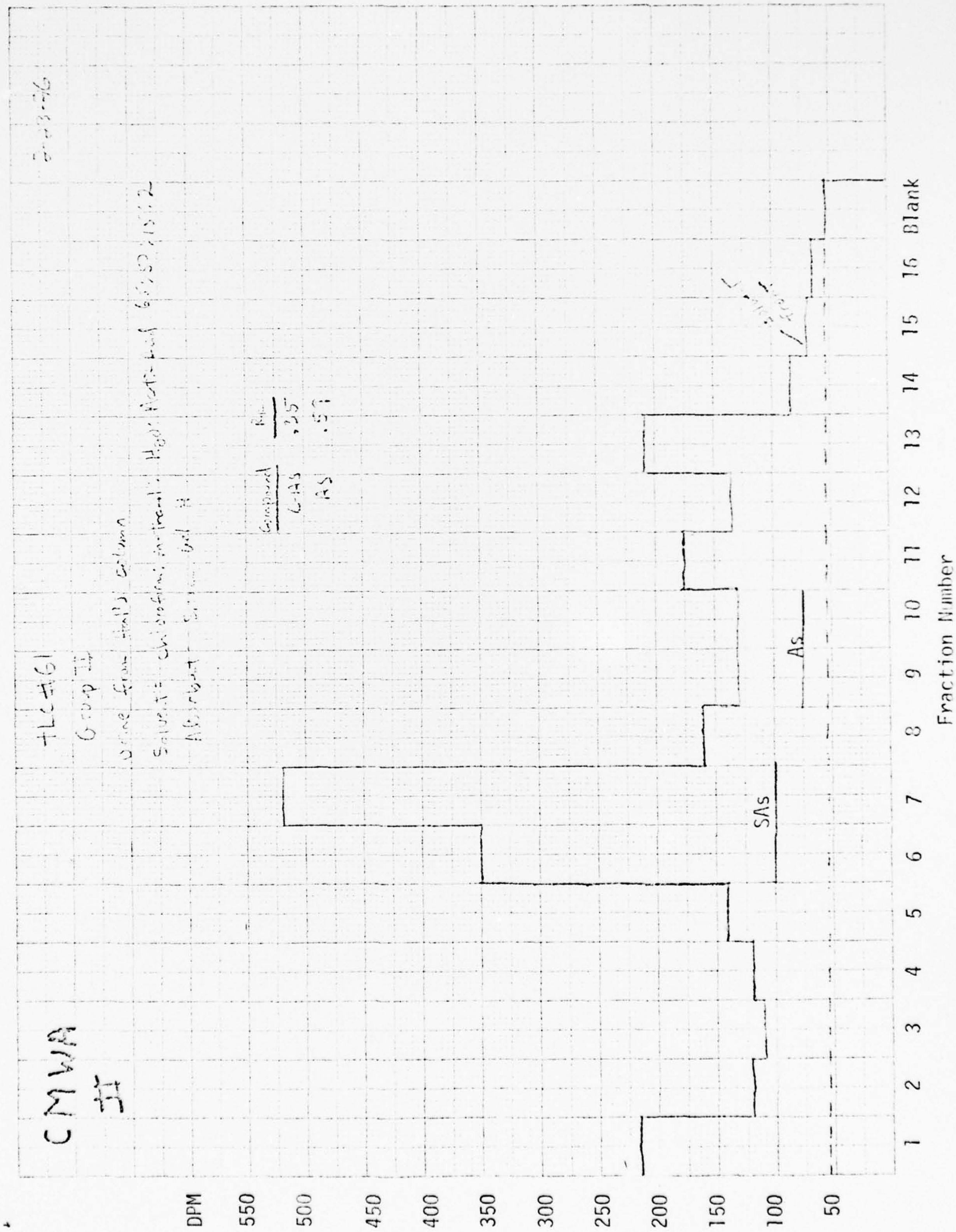
Figure 7.

Figure 8.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Blank
Fraction Number

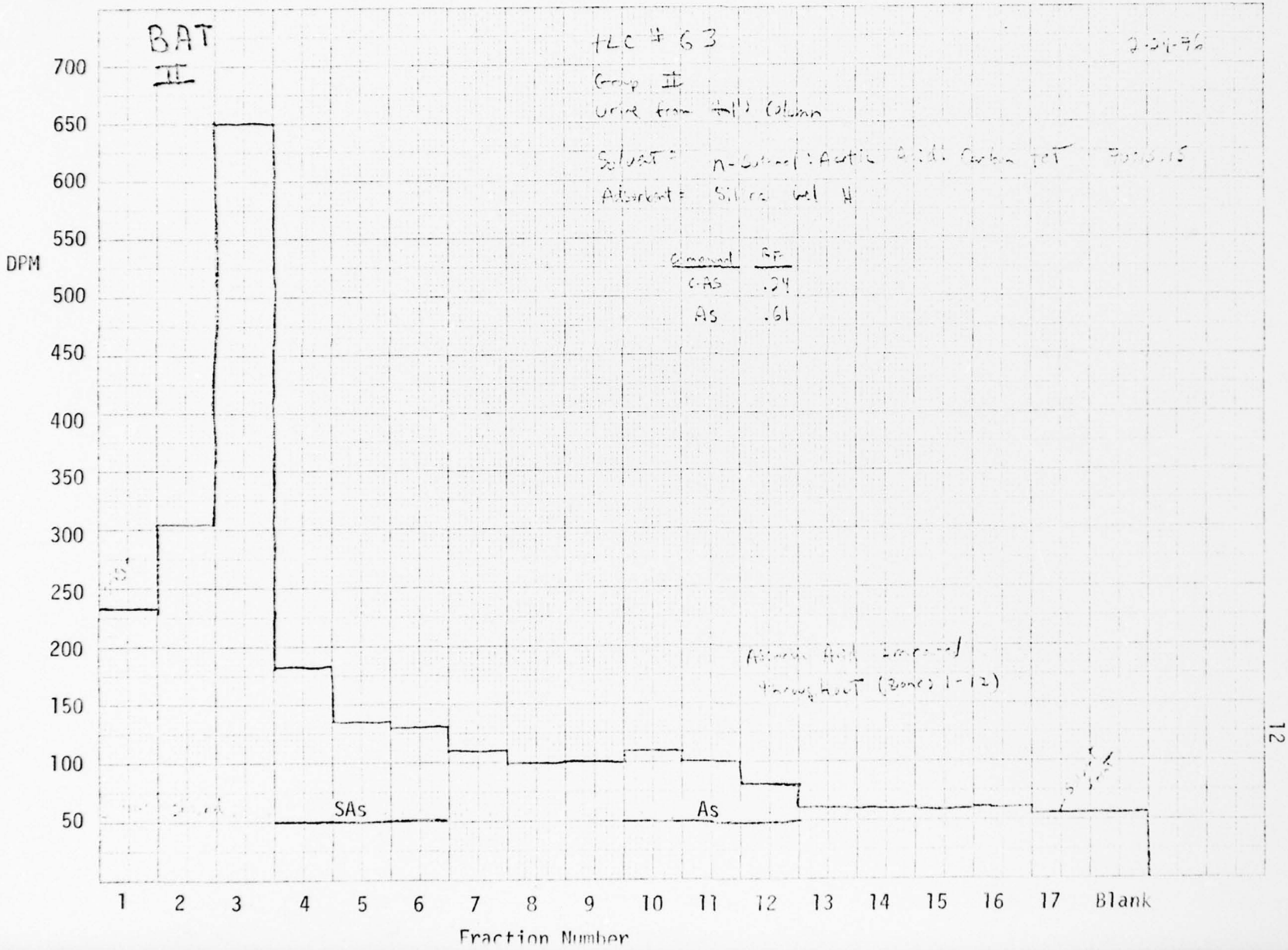

BAT
II
TLC # 63
2-24-76
Group II
Urine from Hill Column
Solvent: n-Butanol : Acetic Acid : Carbon Tet : Water
Absorbent: Silica Gel H
Compound RF
C-AS .24
AS .61
Acetic Acid Compound throughout (Zones 1-12)
SAs
As
DPM
700
650
600
550
500
450
400
350
300
250
200
150
100
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 Blank
Fraction Number

Figure 9.

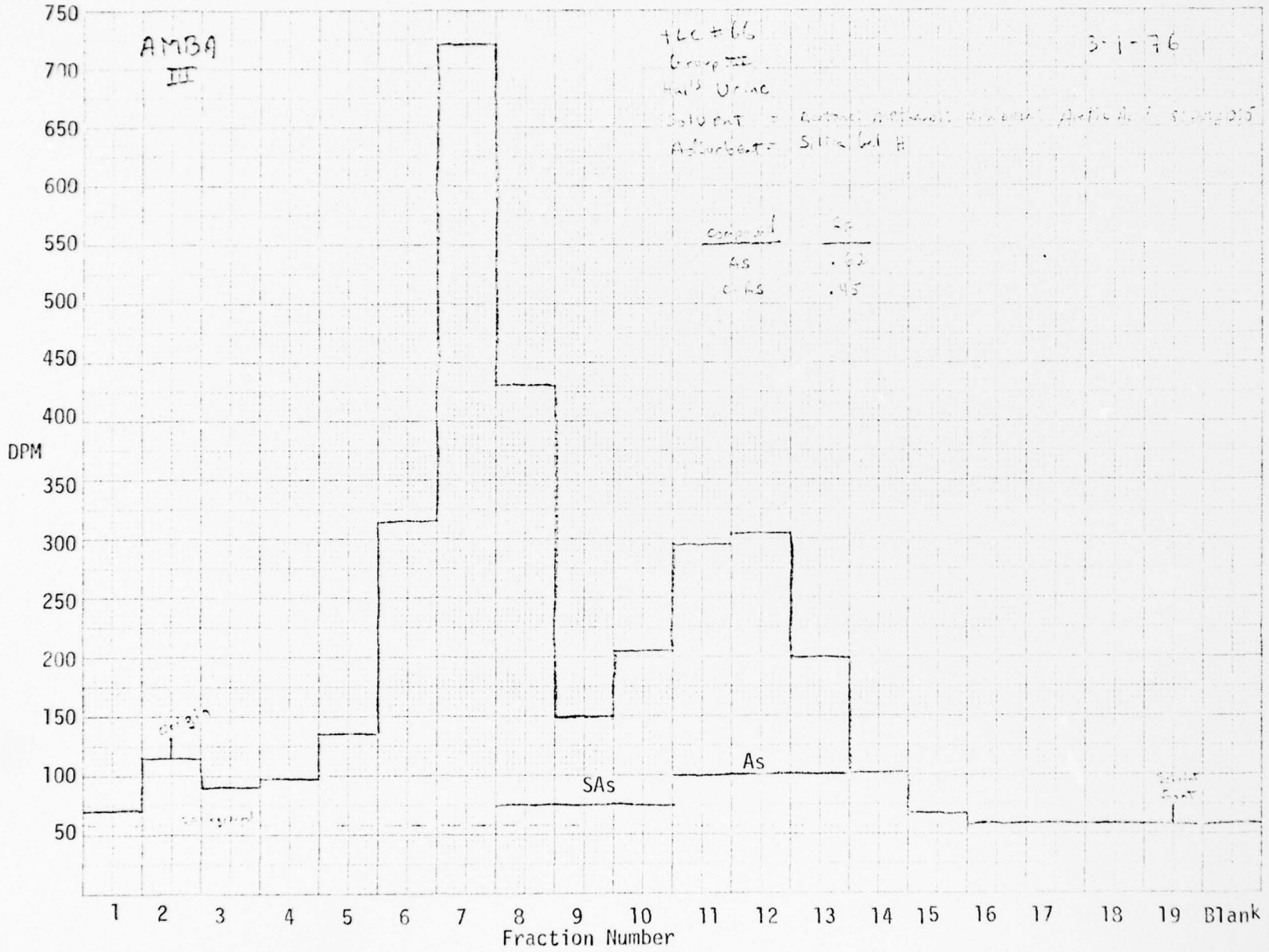

AMBA
III
TLC #66
Group III
Man's Urine
3-1-76
DPM
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
SAs
As
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 Blank
Fraction Number

Figure 10.

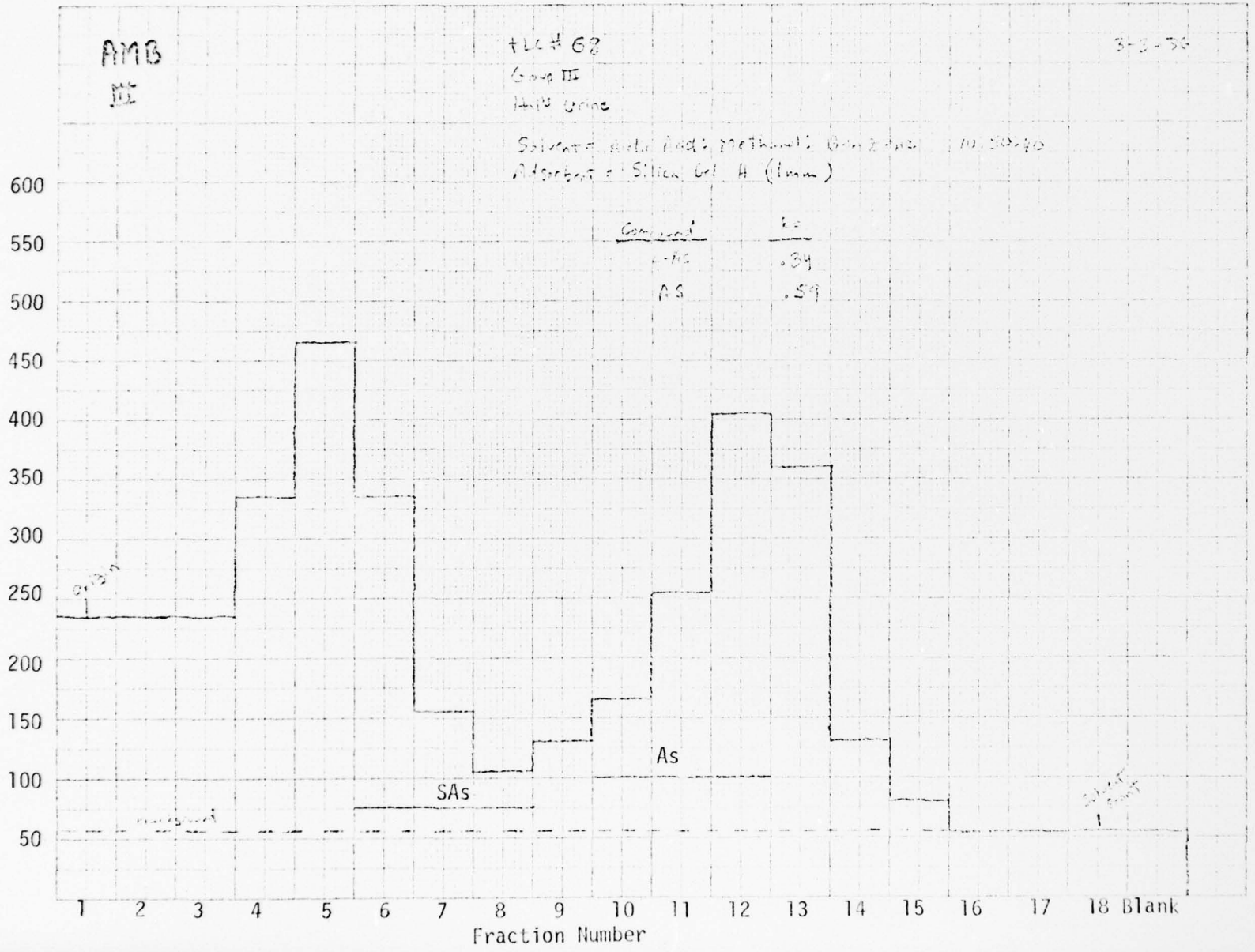

AMB
tlc # 68
Group III
Human Urine
3-3-56
Solvent: Acetic Acid: Methanol: Benzene (10:30:60)
Adsorbent: Silica Gel A (1mm)
Compound
Rf
AS
.59
600
550
500
450
400
350
300
250
200
150
100
50
As
SAs
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 Blank
Fraction Number

Figure 11.

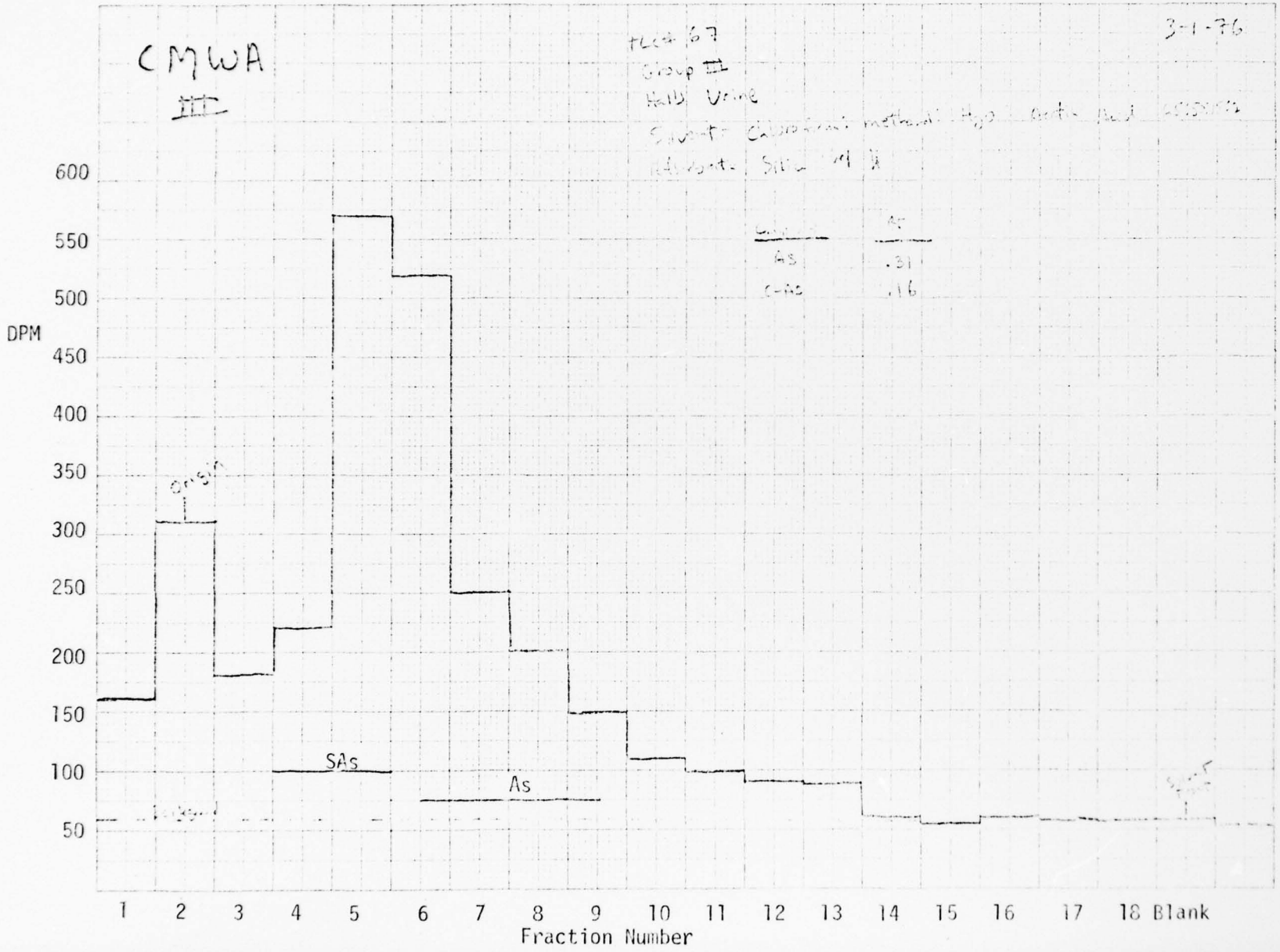

CMWA
III
3-1-76
600
550
500
450
400
350
300
250
200
150
100
50
DPM
SAs
As
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 Blank
Fraction Number

Figure 12.

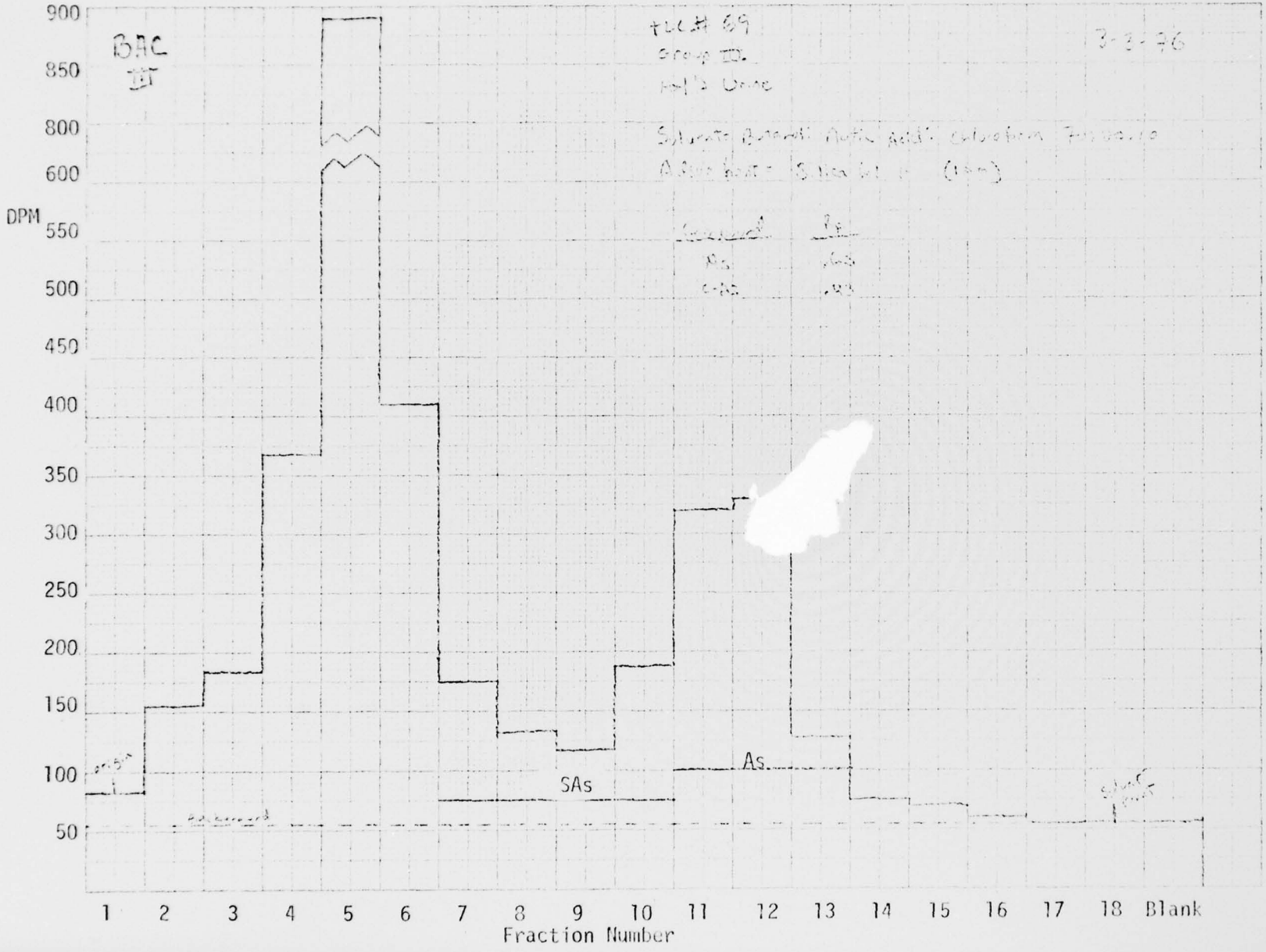

DPM
900
850
800
600
550
500
450
400
350
300
250
200
150
100
50
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
Blank
Fraction Number
SAs
As

Figure 13.

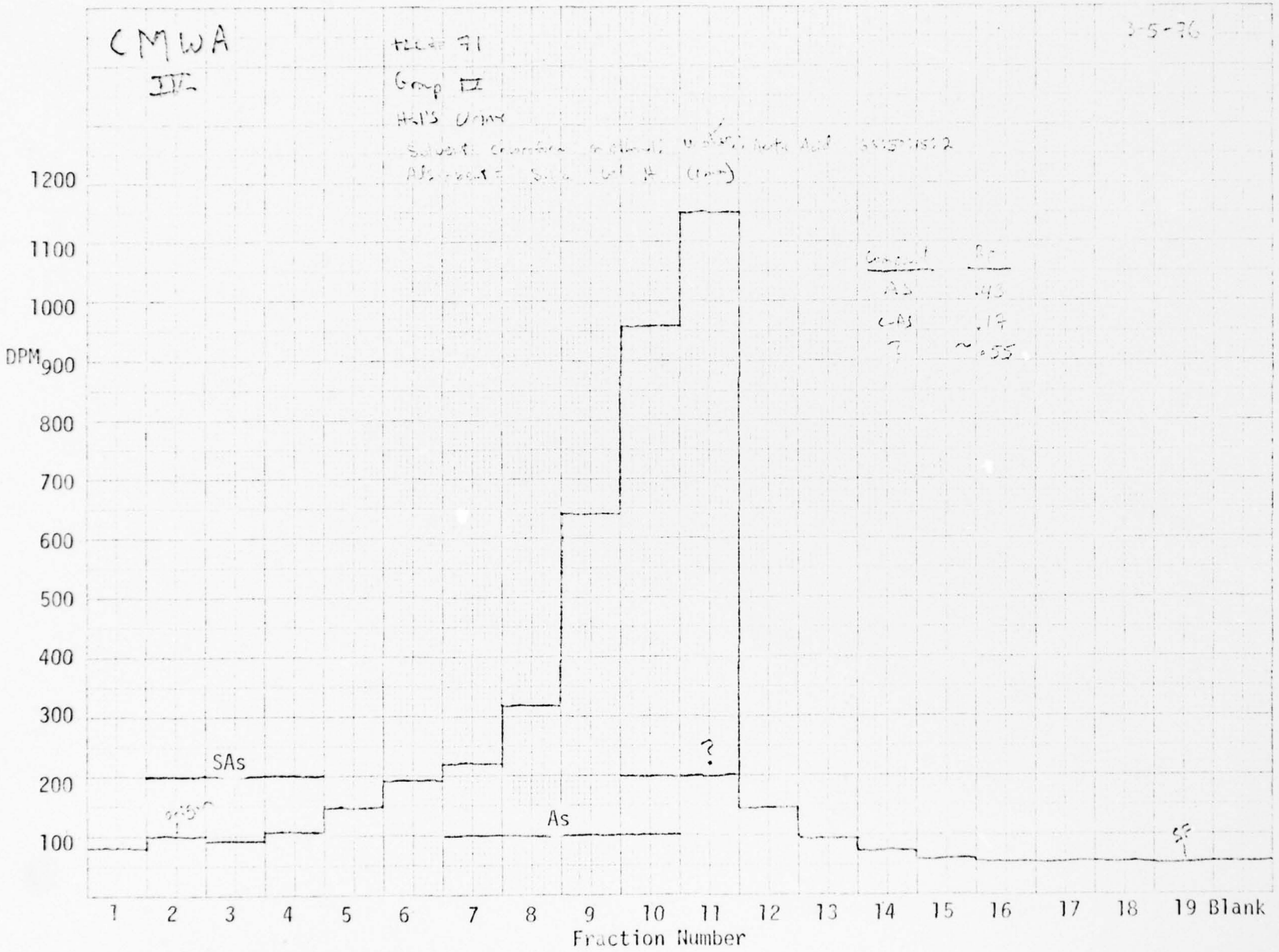

CMWA
III.
3-5-76
1200
1100
1000
DPM 900
800
700
600
500
400
300
200
100
SAs
As
?
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 Blank
Fraction Number

Figure 14.

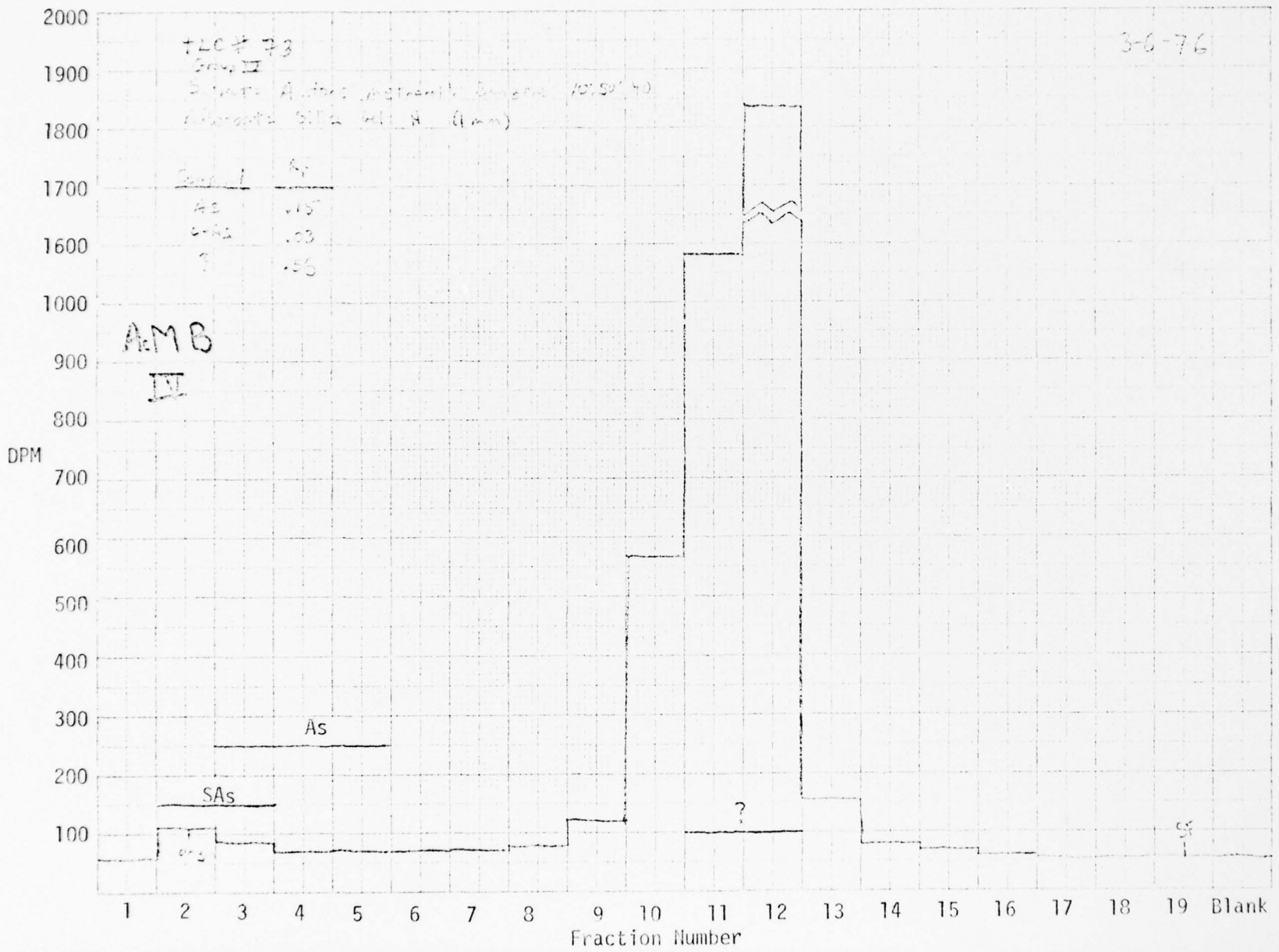

2000
1900
1800
1700
1600
1000
900
800
DPM
700
600
500
400
300
200
100
AMB
II
As
SAs
?
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 Blank
Fraction Number

Figure 15.

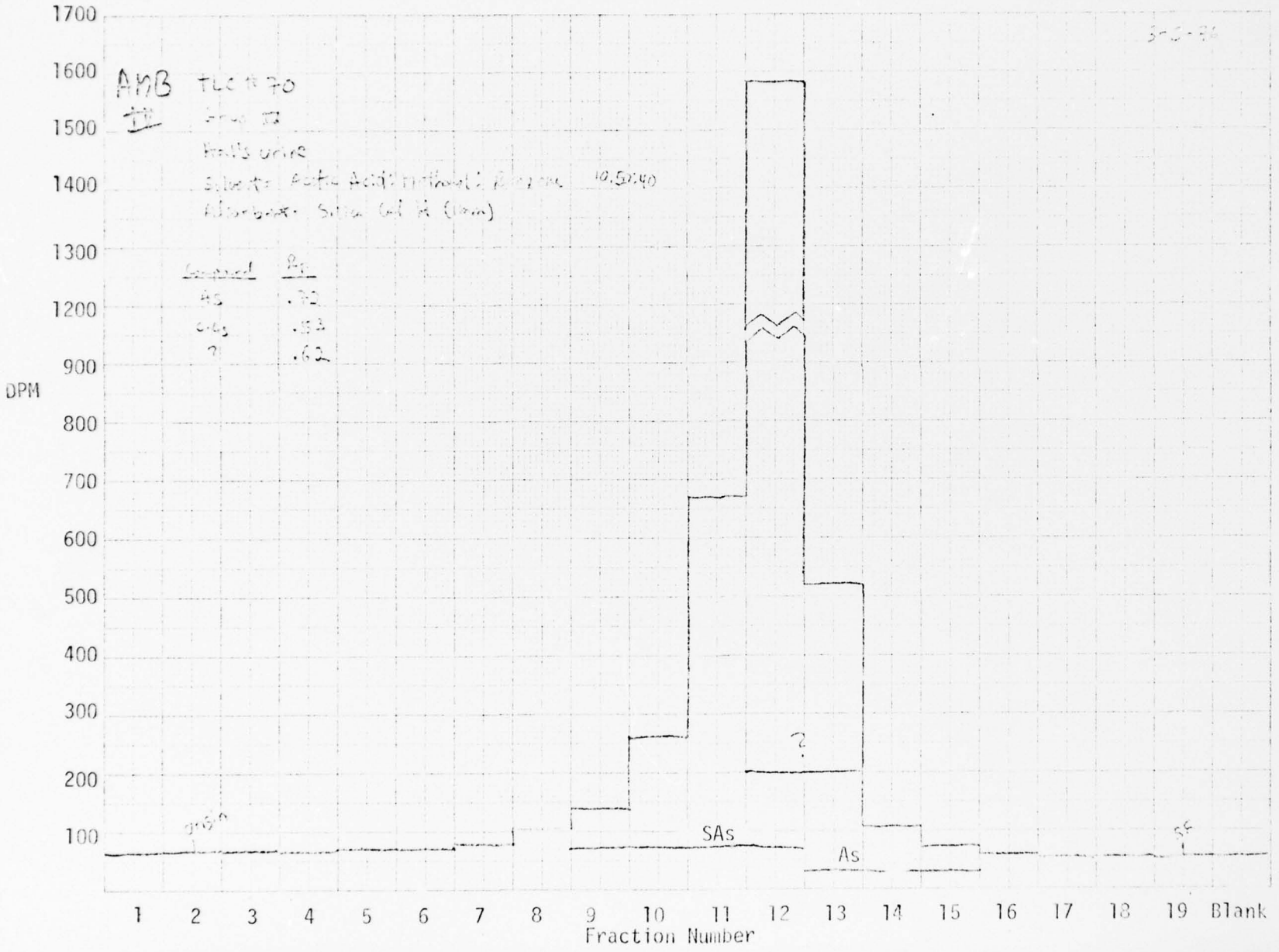

AMB TLC #70
Solvent: Acetic Acid:Methanol:Benzene 10.5:10:40
Absorbent: Silica Gel H (1mm)
Compound Rf
AS .72
unk .52
? .62
DPM
1700
1600
1500
1400
1300
1200
900
800
700
600
500
400
300
200
100
SAs
As
?
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 Blank
Fraction Number

Figure 16.

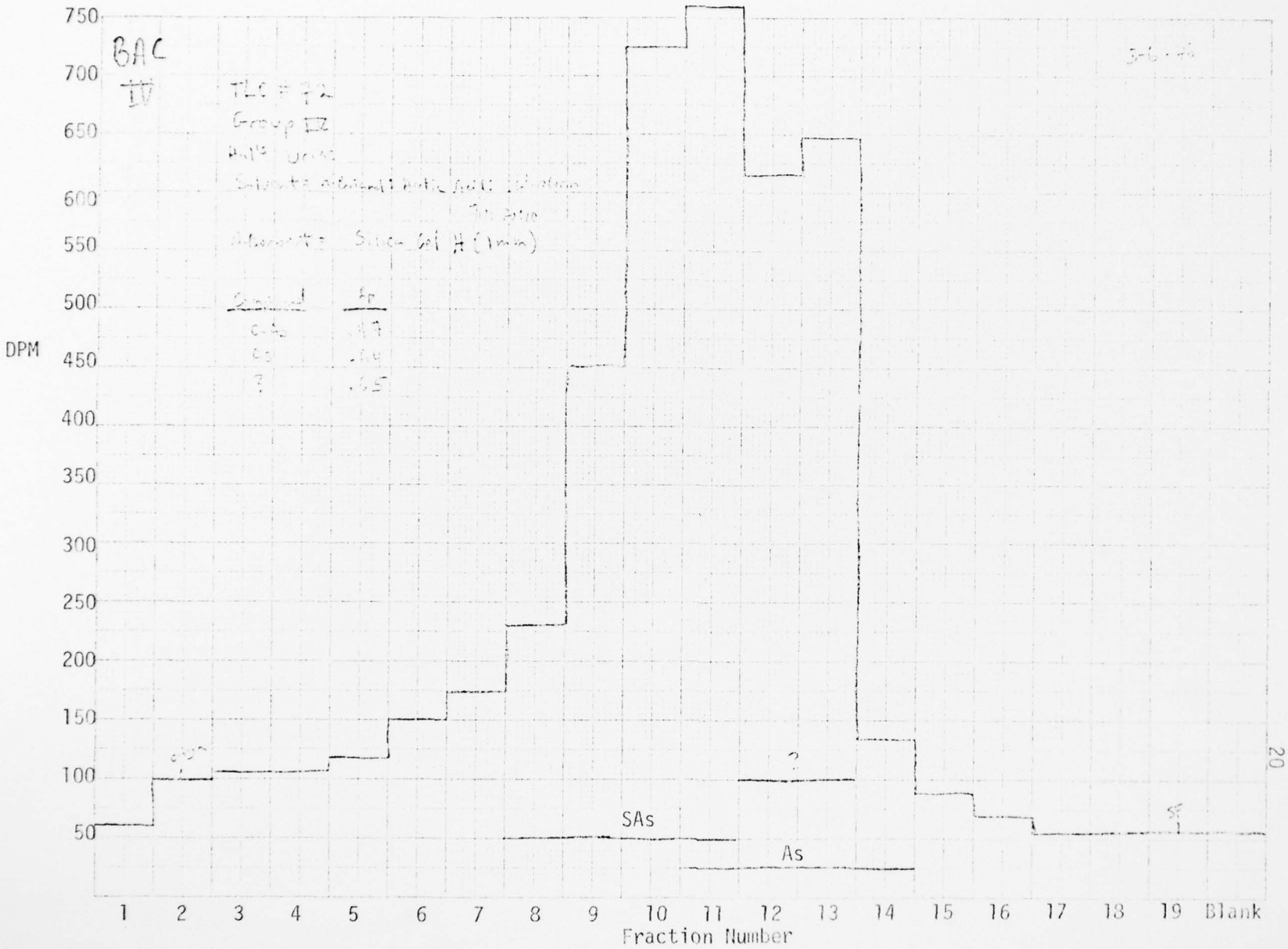

DPM
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
SAs
As
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 Blank
Fraction Number

Figure 17.

Discussion

There are a number of questions that this data could shed some light on, the first being the number of radioactive compounds present in the urine. By looking at the four graphs for groups I-IV with CMWA as a solvent (Figures 5, 8 12 and 14), one can see a minimum of six and a maximum of nine. Group I shows one compound at the origin, and another in zones 6 through 9. Group II also shows one at the origin, which may be the same as that in Group I. Group II shows one in zones 5 and 6, one in zone 10, and one in zone 13. Group III shows 1 at the origin, and 1 in zones 3 through 7. The latter may be overlap from the second peak in group II. Finally, group IV shows 1 compound in zones 7 through 10. It is interesting to note that the compound in Group IV appeared as a yellow splotch on the TLC plate in this, as well as other systems.

Using this same analysis technique, the AMBA data seems to show a minimum of 4 and a maximum of 6 (substituting one of the other systems for the one missing here for group IV). The other systems are rather sketchy, but seem to confirm that there are between 4 and 10 compounds. As mentioned before, this data is inconclusive, but does put one in the right ballpark.

The second question posed when looking at this data concerns the presence of SAs. When SAs is put on the DEAE-32

sulfate column, it is eluted in the vicinity of group II. The data here shows a correlation between a radioactive hump and the SAs standard in the CMWA system, Group I (Figure 5). The AMBA system (Figure 6) shows radioactivity in that area also, so we cannot disconfirm its presence in Group I. For the Group II data, there are nice correlations in the CMWA and AMBA (Figures 7 and 8) systems, and no disconfirmation in the BAT system (Figure 9). Group III data shows one correlation in the CMWA system (Figure 12), though a poor one, and near misses in the other three systems (Figures 10,11, and 13). Group IV data shows correlations in the AMB and BAC systems (Figures 15 and 17), but also shows two nice disconfirmations in the AcMB and CMWA systems (Figures 14 and 15).

On the basis of this data alone, one could suggest that SAs is present in Groups I and II. The fact that it comes out in Group II on the column helps determine that it is not present in Groups III and IV. However, it may be that there is, indeed, some overlap in Group I. One should note again that these conclusions are somewhat sketchy. The lack of good data here is compounded by the possibility that high ion concentrations in the urine are interfering with the results. This problem is dealt with in Part II (see page 26).

The last bit of discussion here will concern the nature of these compounds on the basis of their chromatographic properties. One should recall that these compounds all came off a DEAE-32 column (a weak base) with a very dilute $SO_4^=$ solution, and none came out in the void volume, so they are all weak acids, or neutral compounds. This would be expected if they are derivatives of ascorbic acid.

Speculations are more difficult from the TLC data. One must recall that silica gel is a powerful adsorbent, and that elution will take place largely due to the polarity of the compound in question. In general, it seems that the more polar solvents (CMWA, AMB, and BAC) gave the larger R_f values. In addition, it seems that acetic acid was a necessary component in these systems to move anything. One could say, then, that these compounds are being adsorbed quite strongly on the silica gel. This would also be expected if the compounds are As derivatives. It is interesting to note that these compounds all behaved similarly on the column and on the plates. This lends additional support to the theory that these compounds are all ascorbic acid derivatives.

The question as to what these derivatives are is near impossible to answer. On the basis of where SAs and As-2-SO_4

are eluted on the column, one could say that Group III and IV compounds are stronger acids than SAs and weaker acids than As-2-SO_4. However, our conclusions are limited to that. A variety of derivatives have been made (3), and it is only a matter of time before these unknowns are elucidated.

PART II: COLUMN CHROMATOGRAPHY

A number of columns were experimented with before a suitable one was found for our purposes. Each one will be discussed briefly here before continuing with the successful one. The first column tried was an AG-1 resin in the ammonium carbonate form. It was thought that a strongly basic column would give a good separation. Difficulties arose when it was found that As and SAs are unstable at the column pH (~9) and decompose before elution can take place.

The second column tried was also an AG-1 resin, but in the formate form. Formate was chosen because of its ease of removal. It was soon found that almost 5 M formic acid was needed to push SAs off the column, so it was decided to go to a weaker resin.

DEAE-32 in the formate form was the next try, but did not seem to give good separations. Since the elution

concentrations were in the right range, ammonium formate was tried, and found to give good separations. The best were obtained when the pH was adjusted to 3.5. A good characteristic of ammonium formate here is that it can be lypholyzed off if desired.

Methods and Materials

Preparation of the Column: The resin was prepared according to the instructions in the Whatman pamphlet "Advanced Ion Exchange Celluloses" using step 3 under "Equilibration". .5 M ammonium formate was used as a buffer. It was degassed, and then poured into a 30 X 1 cm. column using a large glass tube as a pipette. The resin was rapidly pumped down, and topped off as needed to keep the column full. It was then rinsed with double distilled water to a constant pH (~5).

Equilibration of the Column: .5 M ammonium formate, adjusted to pH = 3.5 was used as a stripper to remove impurities from the column. 50 ml. was found sufficient in most cases. This was followed by a double distilled water rinse until the pH was constant.

Preparation of the Urine: Urine was prepared by passing it through a diaflo membrane under 45 p.s.i. N_2. It was then blown down under more N_2 at 37°C to .5 ml.

Preparation of the Urine without Cl^- Ions: Urine can be prepared with most of the Cl^- ions removed if interference is encountered. The silver formate used to accomplish this can be prepared as follows(4):

> Mix silver nitrate to a 1% solution and precipitate it with 50% NaOH. Spin the solution down, and wash the precipitate with water. Then dissolve the precipitate in formic acid diluted to pH = 2.

This silver formate solution can be used to precipitate Cl^- ions from the urine after it has been filtered through the diaflo membrane. A centrifuge is helpful to spin down the cloudiness between subsequent additions of the silver formate solution. Add the solution only as long as cloudiness appears, since extra Ag^+ ions in solution are undesireable. The urine may then be blown down at 37°C to 0.5 ml.

Running the Column: The column was run at a speed of 0.5 ml./min. Fractions were collected every five minutes. The gradient was 200 ml. of degassed ammonium formate from a concentration of 0 to 0.5 M at a constant pH = 3.5, followed by 50 ml. of stripper (0.5 M ammonium formate at pH= 3.5). The sample was applied, and the gradient begun immediately The UV scanner was also turned on and zeroed. It was sometimes necessary to increase the pump speed slightly near the end of the gradient to prevent

the column from running dry.

Liquid Scintillation Counting: The solutions used were the same as those for the TLC plates (see page 5). The dioxane was again found to be a much superior solvent. Solubilizer was usually needed, and 1-3 ml. of Dr. Tolbert's solubilizer was sufficient. The solutions were counted for ^{14}C and ^{3}H, and efficiency tests were done to correct the data.

Data

The data will again be presented in the form of graphs, plotting DPM versus fraction number. Peaks that have been identified will be labeled as such. The first run was done with 10 ml. of urine, and did not have the Cl^- ions removed. As, SAs, As-2-SO_4, and SAs-2-SO_4 were added as cold carriers (see table 3).

Table 3. Spikes added to the first run.

Substance	Amount Added	Recovery Zones
As	0.25 mg.	4-5
SAs	0.3 mg.	6-7
SAs-2-SO_4	0.2 mg.	27-28
As-2-SO_4	2.0 mg.	35-36

The UV scan obtained showed a variety of absorbing compounds present (see figure 18).

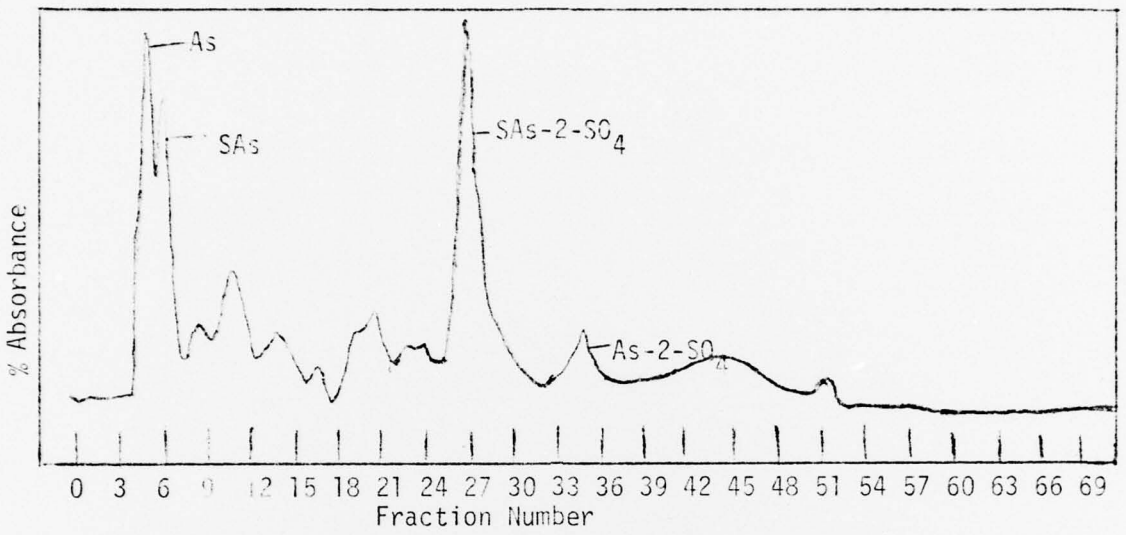


Figure 18. UV Absorbance from run # 1.

The second run was done with 5 ml. urine which was treated to remove the Cl^- ions. This was spiked with 1 mg. SAs. It was recovered in zones 9-15. The correlation in this run is quite spectacular (see Figures 19 and 21).

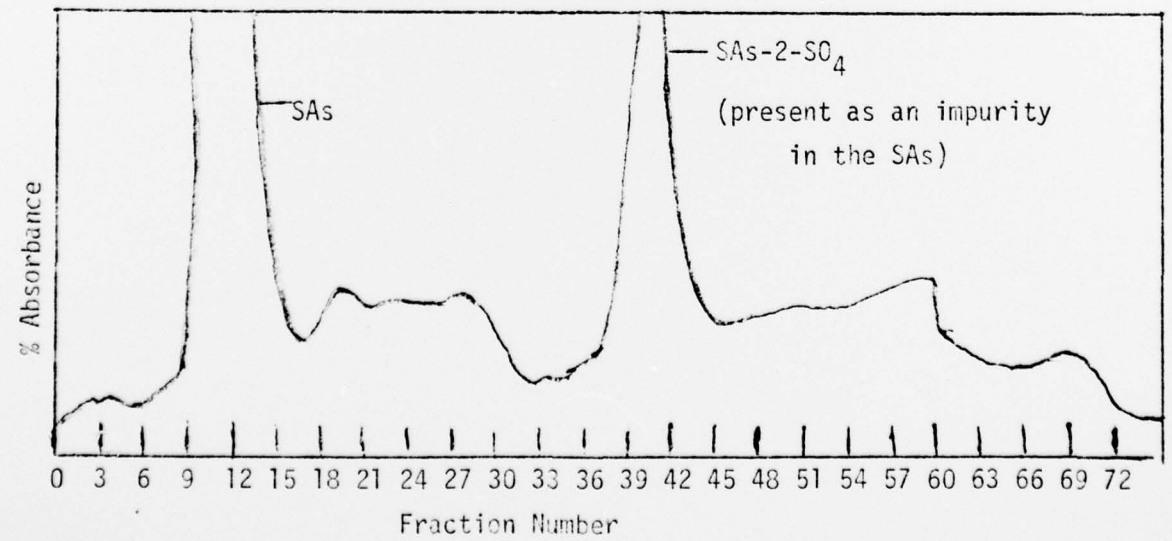
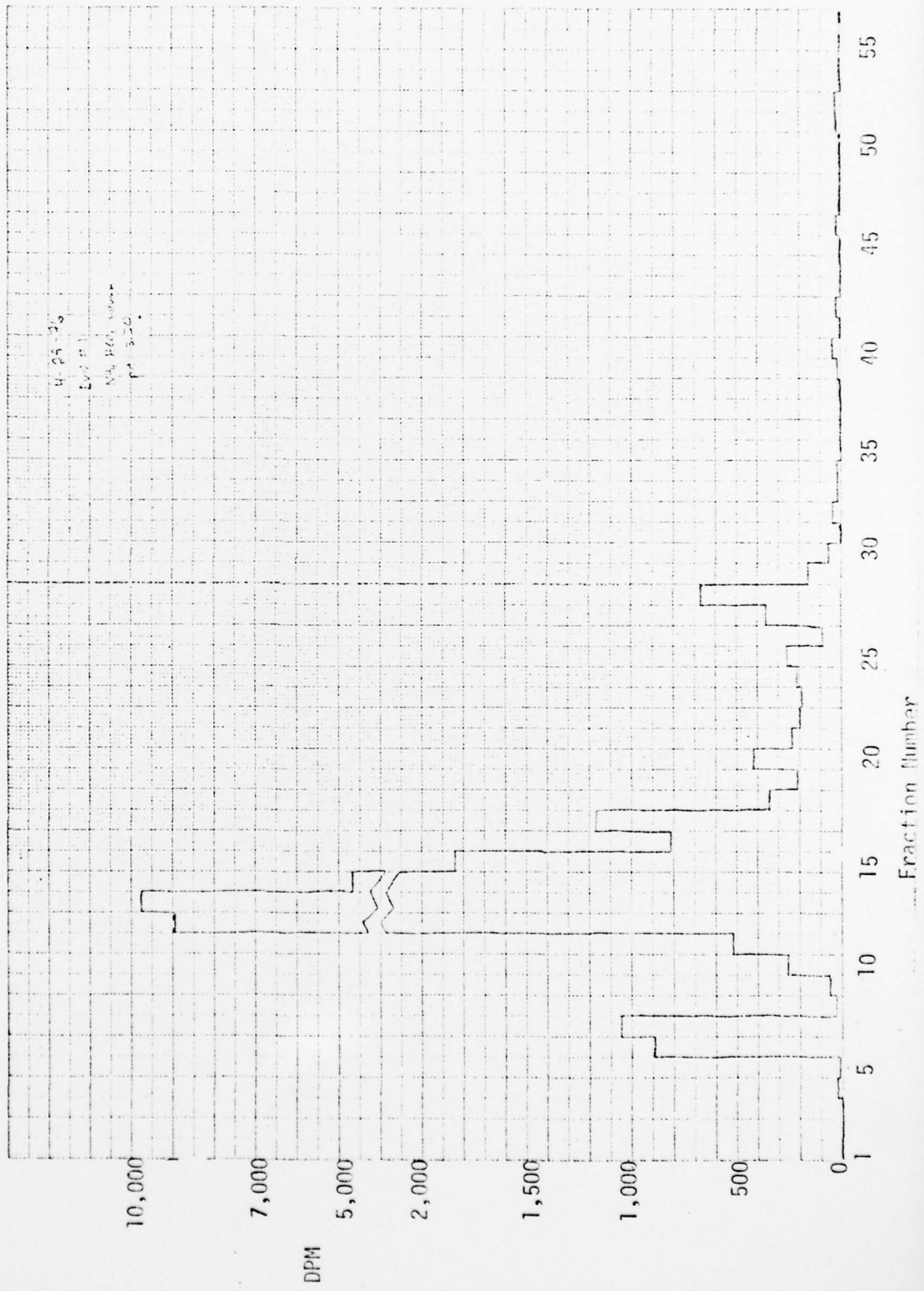


Figure 19. UV absorbance for run # 2.

Figure 20. Run # 1 graph.



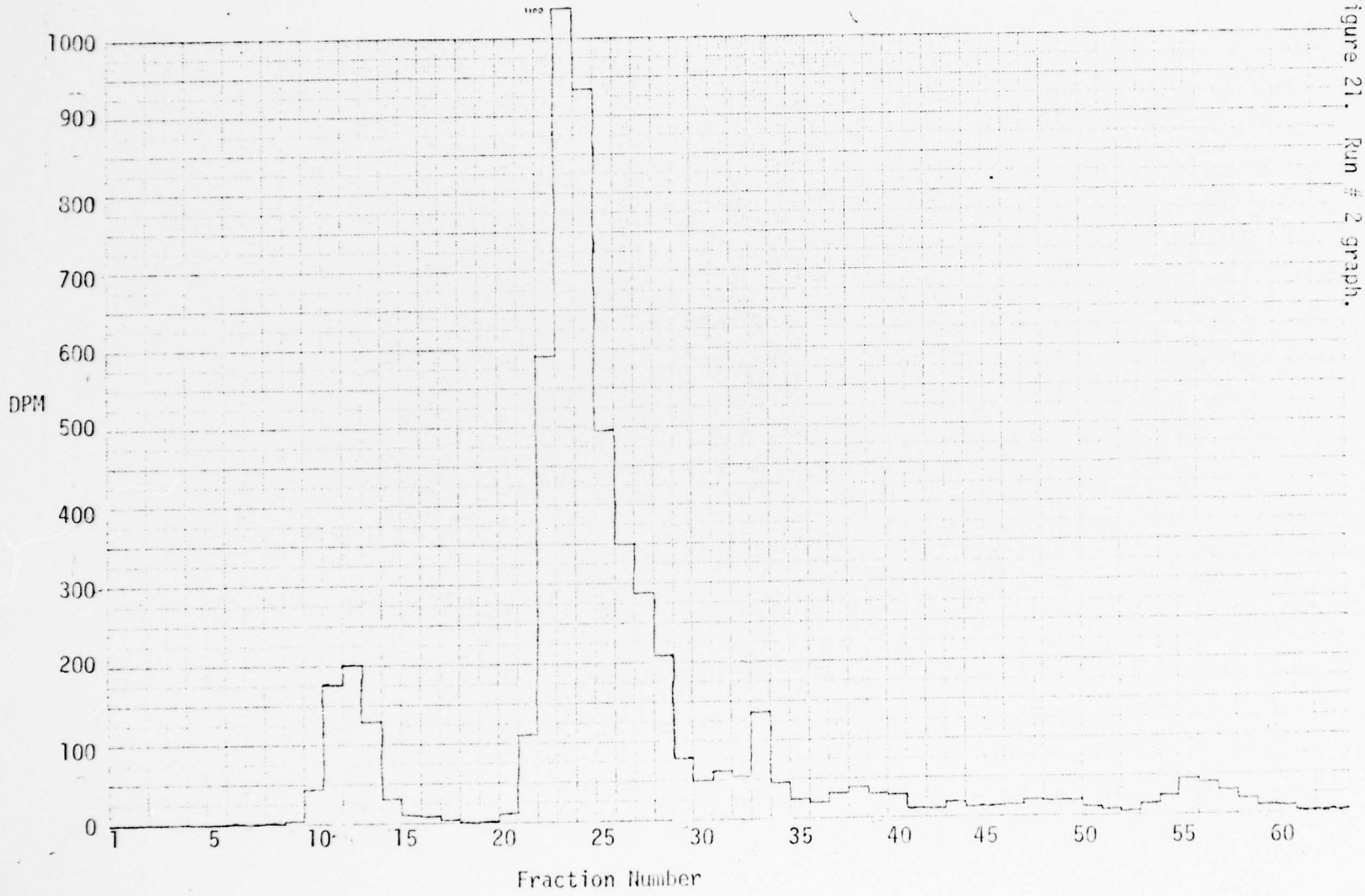


Figure 21. Run # 2 graph.

Discussion

There are two nice correlations between SAs spikes and radioactive peaks, which add considerable support to the theory that SAs is present in the urine as a metabolite of As. In run number 1 the spike was recovered in zones 6 and 7 (Figure 18, Table 3) and a radioactive peak was found in zones 6 and 7 (Figure 20). In run number 2 a spike was recovered in zones 9 through 15 (Figure 19), and a radioactive peak was found in zones 10 through 15 (Figure 21). So there is undoubtedly a compound in urine that behaves chromatographically like SAs.

It appears that each of the radioactive peaks that are believed to be SAs comprises about 9% of the total radioactivity, so it is present in a significant amount. It may be that more was present originally, and that it decomposed. On the other hand, it is also possible that the 9% SAs is the degradation product of another metabolite.

One may ask next: What does the large radioactive peak consist of? It is most likely the major metabolites of As, though we cannot discard the possibility that it may be decomposition products of the major metabolites. According to the TLC data, it may consist of 4 to 6 different compounds. They almost surely contain the As ring, since they behave so similarly

to As on the column. There are a variety of derivatives made that could be candidates for this peak(3).

It is interesting that the UV scans of both runs (Figures 18 and 19) show a variety of UV absorbing compounds. This is to be expected. Only the large proteins have been removed from our samples, and the urine contains a variety of bodily discards that would absorb UV light.

Conclusions

It is difficult to make any solid conclusions based on this evidence alone, but one can determine with strong certainty that SAs is present as a urinary metabolite of As. There has also been some light shed on the other radioactive material present. It is probably a group of metabolites or decomposition products exhibiting weak acid properties. The chances are good that the As ring is still intact, since ring breakdown would change its chromatographic properties drastically. More studies, however, need to be conducted to answer these questions.

There are several experiments that could be done to further elucidate the SAs problem. The first, which will be done in the lab this fall, is to isolate the radioactive SAs from the appropriate fractions, mix it with non-radioactive SAs of good purity, and recrystallize it to a constant specific activity. This will show, with great certainty, that SAs

really is what we are looking at. It will not, however discard the possibility that SAs is only a decomposition product, or otherwise unimportant.

An experiment to demonstrate the importance of SAs would be to isolate radioactive SAs from the liver or brain of a guinea pig injected with labeled As. This would show that SAs is present in the body, and not just as a urinary metabolite.

A third experiment, which would show SAs present in the urine beyond any shadow of a doubt, would be to isolate enough of it in a pure form to run an NMR or mass spectra on it. This may prove difficult, though, as large amounts are required, and studies are still being done on a small scale basis. In any case, further experimentation in this area should prove to be exciting and rewarding.

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